COMPARATIVE STRUCTURE/FUNCTION STUDIES OF *Erwinia chrysanthemi* EC16 PECTATE LYASES

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COMPARATIVE STRUCTURE/FUNCTION STUDIES OF *Erwinia chrysanthemi* EC16 PECTATE LYASES

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Colonization of plant tissue by the phytopathogen *Erwinia chrysanthemi* EC16 is aided by the activities of the pectate lyase isozymes (PLs) which depolymerize the polygalacturonic acid component (PGA) of plant cell walls. The bacterium secretes four pectate lyases (PLa, PLb, PLc and PLe) encoded by structural genes found in two clusters on the bacterial chromosome. Sequence identity between members of the same cluster is high (62% for PLa and PLe and 84% for PLb and PLc), a trait that is not shared across clusters (31% sequence identity shared by PLc and PLe). PLc and PLe have been shown to fold into a similar domain motif, the β-helix. To understand the rationale behind the evolution and retention of these isoforms, the functional differences between PLb and PLc as well as the structural differences between PLs b, c and e were examined. PLb and PLc were found to possess similar affinities for the catalytically required metal cation Ca^{++}. While both depolymerized pectic substrates to the same extent, PLb more readily depolymerized calcium cross-bridged PGA molecules and PLc more readily depolymerized pectins. Pectate lyases B, C and E were found to denature under low concentrations of guanidine hydrochloride, with C_m values of 0.75 M, 1.25 M and 1.75 M, respectively. In thermal denaturation studies, T_m values of 45.6°C, 43.9°C, and 39.7°C were determined for

PLb, PLc and PLe, respectively. No thermal activation of PLe was seen; however, PLb and PLc showed activity maxima of 40°C and 35°C, respectively. Early products of proteolysis were identified by capillary electrophoresis, MALDI-TOF mass spectrometry and high performance liquid chromatography. A shared loop in PLb and PLe was found to be most susceptible to proteolytic cleavage by trypsin and AspN, while a β-strand in PLc was found to be most susceptible to these same proteases. The data demonstrate structural differences among these enzymes that may be the basis for different enzymatic efficiencies under the potential array of environmental conditions experienced by the bacterium. These differences, in turn, may be the basis for the retention of these isozymes as virulence factors, allowing the successful colonization of susceptible plant hosts.

CHAPTER 1
INTRODUCTION

Pectate Lyases and Depolymerization of PGA

The phytopathogenicity of *E. chrysanthemi* EC16 is directly attributed to its ability to produce the extracellular enzymes that are implicated in the maceration of plant cell walls (Collmer and Keen, 1986). An important group of enzymes involved in this tissue maceration is the pectate lyase family of enzymes (PLs). In *E. chrysanthemi* EC16, four functional pectate lyases are secreted that depolymerize polygalacturonic acid (PGA) with a β-eliminative reaction mechanism that cleaves α-1,4-galacturonosyl bonds in the PGA polymer, resulting in formation of Δ-4,5-unsaturated oligogalacturonates (Figure 1).

Genetic Manipulation of *E. chrysanthemi* EC16 and *E. coli* HB101

The *pel* genes are organized in two clusters on the *E. chrysanthemi* EC16 chromosome (Barras and Chatterjee, 1987, Barras et al. 1987, Keen et al., 1984, Keen and Tamaki, 1986). The structural genes for PLa (*pel*A) and PLe (*pelE*) are located in one cluster with the nonfunctional, truncated *pel*D gene, and the structural genes for PLb (*pelB*) and PLc (*pelC*) are located on another cluster. These clusters are not arranged as operons, but rather as independently regulated transcriptional units, with each gene having its own promoter sequence and rho-independent translational termination sequence (Diolez and Coleno, 1985, Reverchon et al., 1986, Tamaki et al., 1988). Primary sequence homologies are high between members of the two gene clusters, with PLa and PLe sharing a 62% sequence identity, and PLb and PLc sharing an 84% sequence identity (Tamaki et al., 1988). It is this degree of homology between the members of each cluster, coupled with the clustering of the structural genes themselves, that has led to the theory that each cluster arose from the duplication of an ancestral *pel* gene with subsequent divergent evolution of the newly formed structural genes (Tamaki et al., 1988).

As part of the attempt to clone and sequence each of the *pel* genes in *E. chrysanthemi* EC16, four high expression plasmids were constructed, each containing a single copy of a *pel* gene (Table 1). These plasmids were used to transform *E. coli* HB101. Periplasmic extracts of the recombinant *E. coli* cultures were found to macerate potato tuber tissue (Tamaki et al., 1988), indicating successful expression of the plasmid genes. The recombinant proteins were analyzed with respect to isoelectric point (pI) and molecular weight of the expressed proteins (Tamaki et al., 1988) (Table 1). These data further supported the theory of gene duplication events in an ancestral *pel* gene giving rise to the modern PLs. The conclusion that PLa and PLe have diverged to a greater extent than PLb and PLc is supported by the differences in pI and molecular weight between PLa and PLe as opposed to the small differences in these same properties for PLb and PLc.

Regulation of Pectate Lyase Genes and Pathogenicity

The pectate lyases are produced constitutively at very low levels unless induced (Collmer and Bateman, 1981, 1982, Tsuyumu, 1977) by a range of environmental signals including: removal of nitrogen limiting conditions (Hugouvieux-Cotte-Pattat, 1992), decreases in temperature, a shift towards anaerobic conditions, decreases in medium osmolarity (Hugouvieux-Cotte-Pattat et al., 1992), and decreased iron concentration

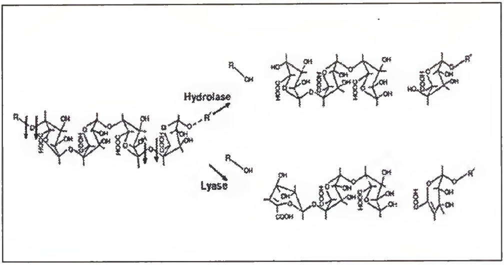


Figure 1: **Reaction catalyzed by endolytic pectate lyases and pectate hydrolases (Preston et al. 1991).**

Table 1: Plasmids used and characteristics of gene products

Plasmid	Structural Gene	Lyase Encoded	pI	Molecular Weight (kDa)
pPEL812	pelA	PLa	4.6	38.7
pPEL344	pelB	PLb	8.8	37.9
pPEL410	pelC	PLc	9.0	37.6
pPEL748	pelE	PLe	9.8	38.1

Plasmids used to transform *Escherichia coli* HB101 were constructed as per Keen et al. 1988. Isoelectric point (pI) and molecular weight information are for the mature form of each enzyme.

(Expert and Gill, 1992). In addition to these induction signals, the addition of cell wall fragments, PGA, isolated oligogalacturonates (Chatterjee et al., 1979, Zucker and Hankin, 1970), or the product of the catabolic utilization of pectate, 2-keto-3-deoxygluconate (KDG) (Hugouvieux-Cotte-Pattat and James, 1996) all serve to increase the expression of the pectate lyase genes to 40-fold higher levels than those seen before the addition of the chemical stimulus. The complexity of the regulation of the *pel* genes serves to underscore the importance of these enzymes in the pathogenic process, and, as such, has been the focus of intense research effort for several years. To date, the picture of the means by which the *pel* genes are regulated is still not entirely clear. As has been previously mentioned, it is known that each of the *pel* genes is a discrete transcriptional unit (Diolez and Coleno, 1985, Reverchon et al., 1986, Keen et al., 1988), which allows for the possibility of distinct control of each *pel* gene. Some evidence for the differential control of the *pel* genes comprising each cluster was provided by the study of two mutant strains of *Erwinia chrysanthemi* B374 (Huguovieux-Cotte-Pattat et al., 1986). One class of mutants, dubbed *cri* for "catabolite repression insensitive," produced and secreted the products of the *pel* genes in the presence of glucose, with PLb and PLc produced at higher concentrations than either of the other 3 PLs. The second class of mutants expressed the *pel* A, D and E genes regardless of growth phase, and were hence dubbed "growth phase independent" (*gpi*) mutants. This study implicated the cAMP receptor protein (CRP) as the first of the four known repressor systems regulating PL synthesis. These suspicions were confirmed by subsequent analysis of the 5' noncoding regions of the pelE, pelB and pelC genes (Gold et al., 1992, Hugouvieux-Cotte-Pattat and Robert-Badouy, 1992), studies that revealed the presence of a catabolite repressor protein binding site in the regulatory region of each gene. A second repressor system involved in PL regulation is the gene product of the *kdg*R locus.

This transcriptional repressor is inactivated upon binding an intermediate in the catabolism of pectic fragments, KDG, allowing for transcription of all functional *pel* genes (Reverchon et al., 1991) as well as the pectin methyl esterase (*pem*) genes, secretory proteins, and the genes necessary for catabolism of pectic fragments. The *pecS-pecM* gene products represent a third known system that regulates not only *pel* synthesis, but also secretion as well as the production of the cellulase EGZ and the protein machinery necessary for secretion (*out* gene products) (Reverchon et al., 1994). These gene products may work in tandem, with PecM known to be an integral membrane protein that may be involved with sensing an as yet unknown external signal and transducing it to PecS, a transcriptional repressor, thereby causing an increase in PL and cellulase expression, as well as the secretory apparatus necessary to give the enzymes access to their respective substrates. The fourth control system, PecT, displays the differential control observed in the *cri* and *gpi* mutant strains (Condemime et al., 1996). The PecT protein has been demonstrated to repress PLc, PLd, PLe as well as one of the minor pectate lyases, PLl, while having no effect on PLa regulation, and activating PLb synthesis.

The degree and complexity of regulatory systems controlling all genes involved in not only pectinolysis, but all aspects of the pathogenic process, would seem to implicate the importance of the *pel* gene products to phytopathogenic species such as *Erwinia chrysanthemi* EC16. This putative importance of the lyases to the soft rot process has been demonstrated (Collmer et al., 1987) by transforming a non-phytopathogenic *Escherichia coli* strain into an organism capable of macerating potato tuber tissue by introduction of the *pelE* gene. The same study established the importance of the *pelE* gene product in the pectinolytic process by comparing an *E. chrysanthemi* EC16 mutant with the ability to produce PLs a, b and c, but not e with the transformed *E. coli* strain. The *E. coli* strain expressing *pelE* was found to macerate more of the tuber tissue than the *E. chrysanthemi* EC16 *pelE*- mutant. These results allude to the importance of PLe in virulence and corroborate previous work (Roeder and Collmer, 1985) in which the virulence of *E. chrysanthemi* EC16 *pelB*- or *pelC*- mutants was undiminished. However, *E. chrysanthemi* EC16 is not just a tuber pathogen, and full virulence in the environment may very well require all of the pectate lyase isozymes, thereby warranting some authors to refer to the lyases as "virulence factors" (Yoder et al., 1993). The nature of this virulence may very well lie in the range of substrates that the organism could rapidly depolymerize to products of sufficient size to allow for uptake and subsequent catabolism, thereby allowing the organism to reproduce within the host plant, directly furthering the process of infection (Reverchon et al., 1991, San Francisco et al., 1996).

Kinetic HPLC Determination of Products Formed by Pectate Lyases a, b, c, e

Previous studies in this laboratory (Preston et al., 1992) have used reverse-phase ion-pair high performance liquid chromatography (RPIP HPLC) to characterize each of the recombinant proteins with respect to the products formed during the depolymerization of PGA (Figure 2). The acidic PLa was found to make random, endolytic cleavages, producing a broad spectrum of products ranging in degree of polymerization (DP) from 2-8, with 56% of the initial substrate depolymerized over the 40 hour study. PLb and PLc were found to be very similar, making a small number of endolytic cuts in the PGA polymer, and then depolymerizing these products to predominantly DP3, with some DP2 and DP4 present at limit. These two enzymes also depolymerized most of the initial PGA over the 40 hour study, with final extents of conversion amounting to 86% and 91% for PLb and PLc, respectively. PLe produced predominantly dimer during the entire time course of the study,

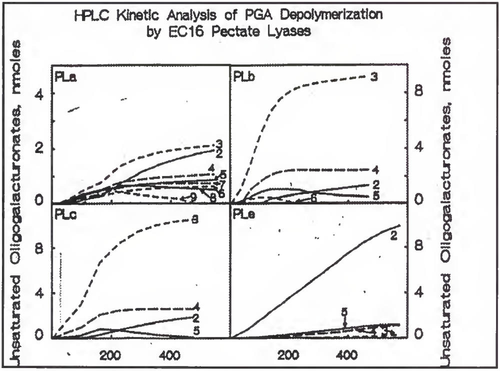


Figure 2: Products formed during the depolymerization of polygalacturonic acid by each of the four pectate lyases secreted by Erwinia chrysanthemi EC16. Adapted from Preston et al., (1992).

with final conversion of over 76% of the initial PGA. These profiles became a sort of "fingerprint" for each lyase, making it easier to distinguish between these similar enzymes.

Structural Studies of Pectate Lyases B and C

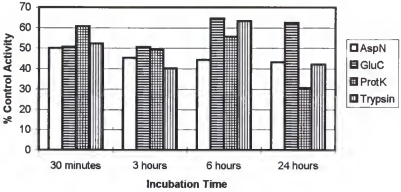
In 1993, pectate lyases c and e were crystallized and structurally elucidated by x-ray crystallography. Both proteins were found to possess a new structural motif, the β-helix (Yoder, Jurnak and Keen, 1993). The β-helical motif consists of 3 parallel β-sheets forming a cylindrical structure with an intrastrand distance of 4.8 angstroms, close enough to allow hydrogen bonding between amino acids of adjacent strands, thus imparting a large degree of structural stability to the molecule. This stability is further enhanced by the presence of several internal structural motifs including: an asparagine ladder, aromatic amino acid stack, aliphatic amino acid stack, and a serine ladder (Yoder et al., 1993).

The secondary structural elements of PLc and PLe were measured by circular dichroism spectroscopy (Sieber et al., 1996), and the β-helical motif was found to give a very distinctive spectrum, with the only apparent differences between each enzyme lying in the intensity of each spectrum. The similarity of both the CD spectra and the molecular models derived from the x-ray crystallographic studies suggests that all pectate lyases share this structural motif.

The structural differences that have evolved between PLb and PLc have been probed by exposing the enzymes to various proteases and monitoring the decrease in enzymatic activity that resulted from proteolytic cleavage of each enzyme as an indicator of the extent of proteolytic digestion (Hurlbert, 1996). Despite the sequence homology of both enzymes and the structural similarity expected for two such homologues, significant differences exist between PLb and PLc with respect to their relative susceptibilities to proteolytic attack (Figure 3). Both PLb and PLc showed similar resistance to the site-specific protease AspN, retaining 43% and 34% of undigested control activities after 24 hours of incubation in the presence of the protease, respectively. When the site-specific proteases GluC or trypsin are used to digest the enzymes, no activity is seen in the PLc sample after 24 hours of digestion, whereas PLb retains 63% of undigested control activity in the GluC sample and 42% of undigested control activity in the trypsin sample for the same 24 hour incubation period. PLc also failed to show resistance to the nonspecific endoprotease ProtK, with all activity relative to undigested control samples removed after only 6 hours of incubation with the protease. PLb, on the other hand, retained 31% of undigested control activity after 24 hours of incubation with the protease under the same conditions. Using MALDI-TOF mass spectrometry, the masses of the peptides produced during the proteolytic reactions were obtained and used to identify regions in the protein that were susceptible to proteolytic attack. Using this technique, putative cut sites for AspN and GluC were located within each molecule. For AspN digested proteins, both enzymes were found to be cleaved at multiple sites within their tertiary structure, but the region near the predicted active site (Asp^{128}, Asp^{159}, Asp^{161} and Asp^{169} in PLb and Asp^{156} and Asp^{162} in PLc) was found to be susceptible in both proteins. In GluC digested samples, only one susceptible peptide bond was found in PLb, that between Glu^{146}-Leu^{147}, whereas multiple sites were found to be susceptible in PLc, with several found in the predicted active site region, namely Glu^{147}-Leu^{148}, Glu^{154}-Cys^{155} and Glu^{166}-Ser^{167}. The location of these putative cut sites is somewhat surprising given the X-ray crystallographic data for PLc and the theoretical model for PLb, placing the scissile bonds within the β-strands that comprise one of the three sheets found within the molecules, not on the more solvent accessible loops and random coil stretches found throughout the periphery of both enzymes.

Proteolysis of Pectate Lyase B

	Lyase Activity			
	30 minutes	3 hours	6 hours	24 hours
AspN	50	45	44	43
GluC	51	51	65	63
ProtK	61	50	56	31
Trypsin	52	40	63	42



Proteolysis of Pectate Lyase C

	Lyase Activity			
	30 minutes	3 hours	6 hours	24 hours
AspN	50	59	43	34
GluC	56	35	43	0
ProtK	61	19	0	0
Trypsin	66	37	25	0

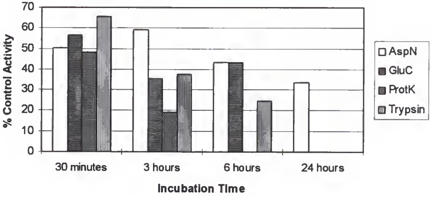


Figure 3: Time course inactivation of pectate lyases B and C during incubation with the proteases AspN, GluC, ProtK and trypsin. Data shown are lyase activities at each sample time relative to undigested control. The ratio of protease to pectate lyase is 1:50. Adapted from Hurlbert (1996).

Given the demonstrated importance of the pectate lyases in the pathogenic process, as well as the differential control systems that seem to have evolved or been retained for each *pel* gene on the *Erwinia chrysanthemi* EC16 chromosome, it would seem that the pectate lyases are not just simple duplications of an ancestral gene that has been retained to increase the amount of protein secreted into the environment. One of the goals of the work presented here is to identify differences in the structure and function of pectate lyases B and C, and thereby provide a rationale for the retention of their homologous structural genes on the bacterial chromosome. In addition to the functional characterizations of PLs b and c, the third and fourth sections of the following work represent attempts to characterize the differences in structure present among 3 of the 4 primary lyases produced and secreted by *E. chrysanthemi* EC16. The studies take advantage of the unique opportunity provided by these particular proteins to explore the effects of naturally selected, site specific changes in amino acid sequence on the relationship between protein structure and function. At the same time, the experiments that follow will provide a better understanding of a novel protein folding motif, as well as offer insights into the advantages afforded the organism by possessing so many isoforms of a single enzyme.

CHAPTER 2
PHYSICAL AND CHEMICAL COMPARISONS OF PECTATE LYASE B AND C

Introduction

The work given in this and all subsequent chapters relies upon the production of quantities of recombinant pectate lyases produced in *Escherichia coli* HB101 transformed with the genes cloned and sequenced from *Erwinia chrysanthemi* EC16 (Keen et al., 1984). In this chapter, pectate lyase B and C preparations were made and purified as previously described (Hurlbert, 1996), and the resulting purified proteins were characterized with respect to calcium affinities, depolymerization kinetics and substrate preference.

The pectate lyase family of enzymes depolymerizes rhamnogalacturonan, a structural polysaccharide found in plant cell walls. The primary cell wall of plant tissues is a complex matrix of polysaccharide polymers, ions and proteins (Dey and Brinson, 1984, Fry, 1986). The polysaccharide fraction can be split into three groups: pectic components, hemicellulose and cellulose. The pectic component is comprised of stretches of α-1,4-linked galacturonans punctuated by rhamnose residues (Fogarty and Kelly, 1983), hence the name rhamnogalacturonan, which serve as branch points and/or attachment points for arabinose moieties. The carboxylic acid group of carbon 6 in the galacturonic acid monomers in rhamnogalacturonan allows for the solution properties of the pectic component to vary quite widely. This group may be methyl-esterified, forming the polysaccharide pectin, or remain free, in which case the polymer is called pectate. In the studies described in this chapter, three types of pectin were used as substrate, which varied in the degree of methylation (DM) of the carboxylic acid groups and compared with PGA.

As seen in Figure 1 of the introduction, the pectate lyases depolymerize pectic substrates in a β-eliminative reaction mechanism, resulting in the formation of a Δ-4,5-unsaturated bond at the non-reducing terminus of the polymer molecule. The introduction of this double bond into the molecule can be followed spectrophotometrically (absorption maximum at 235 nm), allowing the progress of the reaction to be easily monitored (Preston and Rice, 1991). The spectrophotometric measurement of the formation of this bond serves as the basis for many of the techniques used in this chapter, and in this work.

The requirement of pectate lyases for the divalent metal cation calcium has been previously documented (Starr and Chatterjee, 1972), and the affinities of pectate lyases B and C for Ca^{++} have been previously explored (Hurlbert, 1996). In those studies, the lyases were shown to possess quite different affinities for calcium ions, but the ability of the substrate to chelate calcium ions significantly interfered with the assays used, yielding large standard deviations in the experiment sets. In the work described later in this chapter, an observation was made that reduced the observed deviations in the data, thereby providing a reliable determination of the affinities of each lyase for calcium ions.

The ability of polygalacturonic acid to efficiently and tightly bind calcium ions present in solution has been demonstrated (Rees, 1975, Morris et al., 1982). The binding appears to be a cooperative phenomenon and requires a minimum of at least 14 consecutive galacturonic acid residues (non-methylated) to allow cross bridging between two separate PGA molecules. These dimeric structures have been called 2_1 dimers (Rees, 1982) or polymer duplexes and may represent a structural state of the polymer that more accurately reflects the *in vivo* structure. At elevated calcium concentrations, these dimers have been shown to form large aggregates that form a pectate gel. The effect of 2_1 dimerization of pectins and PGA on the activities and extent of depolymerization (conversion) by pectate lyase B and C will be explored in the experiments described in this chapter.

Previous studies used to characterize the pectate lyases have employed PGA (Pfaltz and Bauer)(average DP = 30) and RPIP-HPLC to analyze the reaction products formed during the depolymerization reaction (Preston et al., 1992). Both PLb and PLc were shown to depolymerize the PGA with a high degree of efficiency (84% for PLb and 91% for PLc), with the predominant product detected at limit being Δ-4,5-unsaturated trigalacturonic acid. The similarity in the behavior of each enzyme further supports the gene duplication theory as the basis for the observed homology between the pectate lyase B and C structural genes. These studies do not, however provide any insight into the potential reasons for the retention of two nearly identical structural genes; indeed, they would seem to point towards the redundancy of pectate lyases B and C. Using these studies as a base, further experiments described in this chapter have attempted to define the kinetic parameters for pectate lyase B and C, as well as determine the abilities of each enzyme to depolymerize methylated substrates and the extent of depolymerization of those substrates. These data in turn may provide a glimpse of the evolutionary pressures that have allowed for not only the formation of multiple isoforms of the ancestral pectate lyase, but also the retention of two homologous isozymes in the *E. chrysanthemi* genome.

Materials and Methods

Substrate preparation

Pectin samples (5 g of Bulmer Pectin Fractions A and D, Ref Nos. HT1 and HT4) were oven dried at 40° C under vacuum for 48 hours. Following drying, 500 mg of each pectin type was added to 30 ml of sterile, deionized water with constant stirring at 45°C. Upon solvation, the volume was brought to 50 ml with deionized water and centrifuged at 3000 rpm for 20 minutes at room temperature. The supernatant was poured off and assayed for reducing sugar quantitation (Nelson, 1944) and total uronic acid content (Blumencrantz and Asboe-Hansen, 1973). Polygalacturonic acid was prepared as described in Hurlbert, 1996.

Enzyme preparation and purification

Cultures were grown according to methods previously described (Keen and Tamaki, 1986, Tamaki et al.., 1988, Preston et al., 1992). Batch cultures (1.0 L in 2.8 L Fernbach flasks) of transformed *E. coli* harboring the pPEL344 (PLb), pPEL410 (PLc) or pPEL748 (PLe) plasmids were grown at room temperature with shaking (140 rpm; New Brunswick G10 gyrotory shaker) in Luria Broth containing ampicillin (50 μg/ml). For cultures transformed with pPEL410, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM upon culture initiation. Cultures transformed with pPEL344 or pPEL748 had IPTG added to a final concentration of 1 mM when the culture turbidity (optical density at 600 nm) was 0.5 to 0.7. Cells were harvested at stationary phase (optical density at 600 nm > 2.1) by centrifugation at 5500 rpm (GSA rotor; Dupont-Sorvall) for 15 minutes at 15 °C. Resulting cell pellets were washed with 0.2 M Tris-HCl [pH 8.3], suspended in the buffer solution and centrifuged again under the same conditions used to harvest the cells. Enzymes localized in the periplasmic space were released according to the method of Witholt et al.. Samples of periplasmic enzyme preparations obtained from the Witholt procedure were concentrated to 50 ml in an Amicon stirred cell filtration apparatus (YM-3 membrane, 20 PSI). The samples were then brought to 250 ml with 5 mM Tris-HCl [pH 6.5] and concentrated to 50 ml. This was repeated twice. Final sample volume was approximately 10 ml. All enzyme preparations were purified using a carboxymethylcellulose cation exchange resin (CMC, Whatman CM-52) column system (2.75 x 20 cm). The enzymes were added to the column at a gravity flowrate. After sample addition, 200 ml of 5 mM Tris-HCl [pH 6.5] was run through the column at gravity flowrate, with the effluent collected for lyase activity analysis. The enzymes were eluted from the exchange resin with a 0-0.2 M NaCl gradient (320 ml total elution volume). Fractions (4 ml) were collected during the gradient elution for analysis on a Hewlett-Packard 8451A diode array spectrophotometer. Absorbances of each fraction were taken at 260, 280 and 310 nm using distilled, deionized water as a blank for all wavelengths. Fractions thought to contain lyase were checked for activity using the standard lyase activity protocol: Aliquots of each fraction (10 µl) were added to 500 µl of buffer (100 mM Tris-HCl [pH 8.5], 0.2 mM $CaCl_2$), 200 µl of 0.5% PGA and 290 µl of deionized water. The change in absorbance at 234 nm was monitored on a Hewlett-Packard 8451A diode array spectrophotometer. The spectrophotometer was blanked with all components of the reaction mixture, with 10 µl of deionized water replacing the enzyme aliquot.

Fractions showing lyase activity were pooled and concentrated in an Amicon Centriprep-3 microconcentrator unit (2,000 rpm, 10 °C, 1 hour) with both retentate and filtrate assayed for lyase activity after each centrifugation. When the final volume of the combined fractions was 1 ml, 2 ml of 50 mM Tris-HCl [pH 8.5] was added to the

Centriprep-3 unit, which was then centrifuged again under the conditions given above. This was repeated 3 times, until the final volume of the concentrate was 1.2 ml. Purity was determined using matrix-assisted laser desorption ionization mass spectrometry (MALDI-TOF MS). For MALDI-TOF analysis, a stock solution of 200 ηg/μl of protein was made; 1 μl was then removed and added to 1μl of saturated 3,5-dimethoxy-4-hydroxy-cinnamic acid (sinapinic acid). The sample was mixed on a sample pin, allowed to air dry and analyzed on a Vestec Research H MALDI-TOF mass spectrometer. Protein concentrations were measured using bicinchoninic acid (Smith et al., 1985).

Optimization of calcium ion concentrations for the lyase catalyzed depolymerization reaction

Reactions were set up in 1 ml quartz cuvettes, at 14 concentrations of $CaCl_2$: 0 mM, 0.005 mM, 0.01 mM, 0.05 mM, 0.1 mM, 0.15 mM, 0.2 mM, 0.25 mM, 0.3 mM, 0.35 mM, 0.4 mM, 0.45 mM, 0.5 mM, 0.75 mM and 1.0 mM. To standardize the amounts of enzyme added to each reaction mixture, enzyme stock solutions were prepared based on the activity of each sample in 0.2 mM $CaCl_2$ and 0.1 mM PGA. For each calcium concentration, 2 different initial activities of enzyme were used: 13.0 units and 39.0 units, where 1 unit is the amount of enzyme necessary to produce 1 nanomole of unsaturated product (1 β-eliminative cleavage) in one minute at room temperature. The substrate concentration was held constant at 0.1 mM in all reactions. All reactions were run in triplicate. Reaction mixtures were prepared by adding constant volumes of 100 mM Tris-HCl [pH 8.5] and 1.0% PGA and varying volumes of deionized water and 10 mM $CaCl_2$ stock solution. Upon addition of all reagents, the reaction mixtures were stored at 4° C for 72 hours. Following cold incubation, the reaction mixtures were allowed to equilibrate to room temperature and were subsequently initiated by the addition of enzyme, and monitored spectrophotometrically at 234 nm on a Hewlett-Packard 8451A diode array spectrophotometer (Figure 7).

Kinetic comparisons of pectate lyases B and C

Reactions were setup to a final volume of 1 ml in quartz cuvettes. Both PLb and PLc catalyzed depolymerization reactions utilized 10 mM stock PGA (prepared as per Hurlbert, 1996) at the following final concentrations: 0.01 mM, 0.02 mM, 0.04 mM, 0.08 mM and 0.1 mM. All reactions were buffered in 50 mM Tris-HCl [pH 8.5, and in 0.4 mM $CaCl_2$ for PLb catalyzed reactions and 0.45 mM $CaCl_2$ for PLc catalyzed reactions. Reactions were initiated upon the addition of 13 units of PL to the reaction mixture with subsequent monitoring of the absorbance at 234 nm on a Hewlett-Packard 8451A diode array spectrophotometer (Figure 5).

Effects of calcium ions and substrate concentration on solution structure of substrate molecules

Solutions of each of the pectin types (Bulmer A, D and PGA) in 1 ml of 10 mM sodium phosphate solution (pH 8.5) were made at 3 different substrate concentrations [0.01%, 0.05% and 0.1% (w/v)] and 3 different calcium chloride concentrations (0 mM, 0.1 mM and 0.5 mM). The circular dichroism spectrum of each of the samples was determined from 190 nm to 300 nm on a Jasco-500C circular dichroism spectrophotometer (Settings: 0.5 second time constant, 0.1 nm step range, 50 nm/min scan rate, 5 scans per sample) using a 1 cm pathlength, water jacketed UV quartz cell. The spectra were obtained immediately after addition of the polysaccharide to the sample and subsequent vortexing. After each spectrum was obtained, the sample was removed and placed in a polyethylene microcentrifuge tube, capped, and left to incubate for 24 hours at room temperature. The spectrum of each sample was re-determined for comparison after the incubation period. Prior to all sample measurements, the baseline spectrum of 10 mM sodium phosphate buffer (pH 8.5) was taken for baseline correction. Average spectra from all five scans of each sample were noise reduced using the Jasco J-700 for Windows Standard Analysis software (ver. 1.50.01. Jasco, Inc. 1997) (Figures 3, 4).

Depolymerization kinetics monitored by reversed-phase HPLC

Solutions (8.0 ml) of 0.1 mM PGA in 50 mM Tris-HCl [pH 8.5] at variable calcium chloride concentrations were set up in 15 ml Corning centrifuge tubes and were incubated at 4° C for 48 hours. For reactions involving pectate lyase B, the $CaCl_2$ concentrations used were 0.2 mM, 0.4 mM and 1.0 mM, whereas reaction mixtures that had pectate lyase C added to them were run at $CaCl_2$ concentrations of 0.2 mM, 0.45 mM and 1.0 mM. Following incubation at 4° C, 4 ml of each reaction mixture was filtered through a Millex HV filter (Millipore, Cat No. SJHV004NS, 0.45 µm pore size) into an HPLC sample vial. Each reaction was initiated by the addition of 9 units of enzyme, mixed by vortexing and incubated at room temperature until each sampling time was reached. The chromatographic conditions consisted of a C_{18} RCSS system in line with an 8MB C_{18} 10 µm column contained within a RCM radial pack (8 cm x 10 cm) compression unit, with the mobile phase (10% acetonitrile : 0.1 M sodium phosphate, pH 6.5 : 10 mM tetra-butyl ammonium hydroxide mobile phase) delivered isocratically (Waters M45 solvent delivery system, 1.0 ml/min). Detection was made by continuously measuring the absorbance of the column effluent at 235 nm using a Gilson Holochrome detector with a 1.0 cm pathlength. Manual injections (50 µl) were made every 30 minutes from the 4 ml filtered sample with 20 minute run times. Data were analyzed using the Millennium Chromatography Manager software (ver 1.10, Waters, Inc.) (Figure 13).

Activities of pectate lyases B and C towards pectin substrates with varying degrees of methylation

Using the previously prepared Bulmer Pectin samples, reaction mixtures were set up identically to those used in the depolymerization kinetics study. Reactions occurred at constant pectin concentrations of 0.1% (w/v) in 8 ml of 50 mM Tris-HCl [pH 8.5] buffer. Calcium concentrations used varied depending on the lyase to be added to the reaction. For analysis of pectate lyase B catalyzed reactions, 0.2 mM, 0.4 mM and 1.0 mM $CaCl_2$ final concentrations were used. For analysis of pectate lyase C catalyzed reactions, 0.2 mM, 0.45 mM, 1.0 mM final concentrations were used. Following reactant addition, the reaction mixtures were incubated at 4° C for 72 hours. As those reaction mixtures were incubating, a set of control reactions to determine the baseline activities of each lyase were set up and analyzed using substrate which had not been pre-incubated. This was accomplished by individually preparing 1 ml reaction mixtures in a 1 ml quartz cuvette with the same concentrations from 0.5% substrate, 100 mM Tris-HCl [pH 8.5], 100 mM $CaCl_2$ stock solutions and deionized water. Upon reactant addition, the contents of the cuvette were mixed by inversion, and the reaction initiated by addition of 9 units of lyase. The cuvette was again mixed by inversion and the initial velocity of each reaction determined by measuring the change in absorbance of the reaction solution at 234 nm on a Hewlett-Packard 8451A diode array spectrophotometer (Figure 6). Following incubation, the pre-incubated reaction mixtures were allowed to equilibrate to room temperature, whereupon the reaction was initiated and the initial velocity was determined by measuring the change in absorbance at 234 nm on a Hewlett-Packard 8451A diode array spectrophotometer (Figure 7)

Measurement of extent of conversion of pectin substrates by pectate lyases B and C

Reactions consisting of 0.1% Bulmer A, D pectins or PGA in 1 ml of 50 mM Tris-HCl [pH 8.5], 0.2 mM $CaCl_2$ and 0.1% PGA in 50 mM Tris-HCl [pH 8.5], 1.0 mM $CaCl_2$ were prepared in triplicate, filtered through Millipore HV (0.45 μm pore size) filters and added to 1.5 ml microcentrifuge tubes. An additional set of PGA samples were prepared and allowed to incubate at room temperature for 24 hours. Reactions were initiated by the addition of 10 units of enzyme to each solution. After 24 and 48 hours of reaction, 100 μl of each mixture was removed, diluted to 1.0 ml with deionized water and the absorbance at 234 nm measured on a Hewlett-Packard 8451A diode array spectrophotometer (Table 2), this allowed an easy determination of the continued progress of the reaction. The lyase activity remaining in each sample was also determined by taking 100 μl of the solution remaining after 48 hours and adding it to 900 μl of fresh reaction mixture consisting of 0.1% (w/v) PGA in 50 mM Tris-HCl [pH 8.5], 0.2 mM $CaCl_2$. The change in absorbance at 234 nm was monitored on a Hewlett-Packard 8451A diode array spectrophotometer (Table 3). Upon completion of the 48 hour absorbance readings, the oligogalacturonides present in each solution were separated and quantitated by reversed-phase HPLC under the previously described chromatographic conditions (Table 2). The extent of conversion of substrate to oligogalacturonides was calculated from the HPLC data. Based upon the law of conservation of matter, given that at reaction initiation, 1 mg of substrate was present in the reaction mixture, the total masses of the detected products should equal 1 mg.

Results

Optimization of calcium ion concentrations for the lyase catalyzed depolymerization reaction

Figure 4 shows that the two lyases have only slightly different affinities for the calcium ions. Pectate lyase B shows maximal activity, regardless of enzyme concentration, at 0.4 mM $CaCl_2$, whereas pectate lyase C shows maximal activity at 0.45 mM $CaCl_2$. The standard deviation of the triplicate reactions is much lower than that obtained in previous experiments in which substrate was not preincubated with calcium ions in buffer before addition of enzyme. Both enzymes show a rapid loss of activity at calcium concentrations higher than 0.45 mM $CaCl_2$. This behavior could be due to the formation of calcium-mediated cross-bridged PGA structures that are not readily bound or cleaved by the enzymes.

Kinetic comparisons of pectate lyases B and C

A continuous increase in pectate lyase B activity occurs as the substrate concentration is increased from 0.01 mM to 0.1 mM (Figure 5). These data provide a K_m value for polygalacturonic acid of 0.013 mM. Pectate lyase C on the other hand displays clearly different behavior. The enzymes displays a linear increase in observed activity from 0.01 to 0.04 mM PGA, but at 0.08 mM and 0.1 mM PGA, the observed activities are less than those measured in the 0.04 mM PGA samples. Including these two substrate concentrations in the linear regression line for the plot of the data yields an extrapolated K_m of 5.5×10^{-3} mM for PGA.

Effects of calcium ions and substrate concentration on solution structure of substrate molecules

In the absence of calcium ions, the methylated pectins show a distinct change in molecular ellipticity as the substrate concentration is increased (Figure 6). This effect is best seen in the data for Bulmer A pectin (Figure 6) with a molecular ellipticity decrease of over 5000 deg·cm^2·dmol^{-1} seen at 212 nm as the pectin concentration is increased for 17.9 μM to 179 μM. The data for Bulmer D pectin (Figure 7), which possesses 40% fewer methyl esters per substrate molecule, shows an ellipticity decrease of 500 deg·cm^2·dmol$^-$ at 210 nm as the pectin concentration is increased from 18.3 μM to 183 μM. PGA does not show a change in molecular ellipticity at any wavelength as its concentration is increased from 9.86 μM to 98.6 μM (Figure 8).

The effect of calcium ions on the secondary structure of pectic compounds is seen in Figures 9 - 12. No significant changes are seen in the spectra of Bulmer A pectin as the calcium concentration increases from 0 mM to 0.5 mM (Figure 9). Bulmer D pectin shows a slight change in the observed molecular ellipticity from 195 nm to 210 nm in the presence of 0.5 mM $CaCl_2$ relative to the spectra obtained in 0 mM and 0.1 mM $CaCl_2$ solutions (Figure 10). A slight decrease in molecular ellipticity at 200 nm is observed in PGA when comparing the spectra obtained in the presence of 0 mM and 0.1 mM $CaCl_2$ (Figure 11). This decrease is magnified when the calcium ion concentration is increased to 0.5 mM, and a blue shift is seen for the wavelength of maximum molecular ellipticity. A comparison of the observed molecular ellipticities for 0.01% solutions of each of the polymers in 0.5 mM $CaCl_2$ is found in Figure 12. The differences between each of the samples can be clearly seen, as can peaks that are seen only at this sample concentration at 265-270 nm and 285-290 nm.

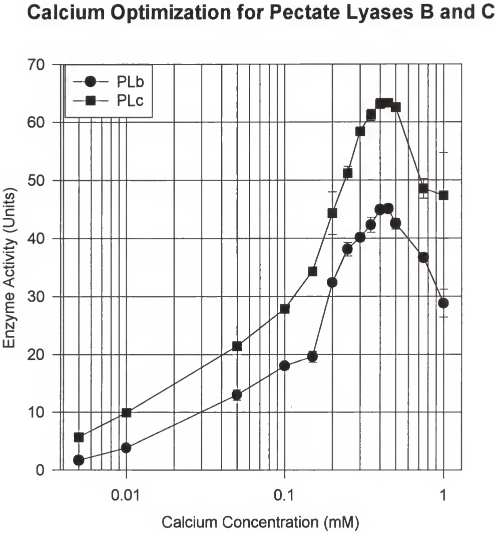


Figure 4: Calcium optimizations of pectate lyase B and C. Data shown are from 1 ml triplicate reactions using preincubated PGA as a substrate. Equivalent amounts of each enzyme (39 U) were utilized in all reactions, based upon enzyme activities measured in non-preincubated reactions consisting of 0.1% PGA (w/v) in 50 mM Tris-HCl [pH 8.5] : 0.2 mM $CaCl_2$.

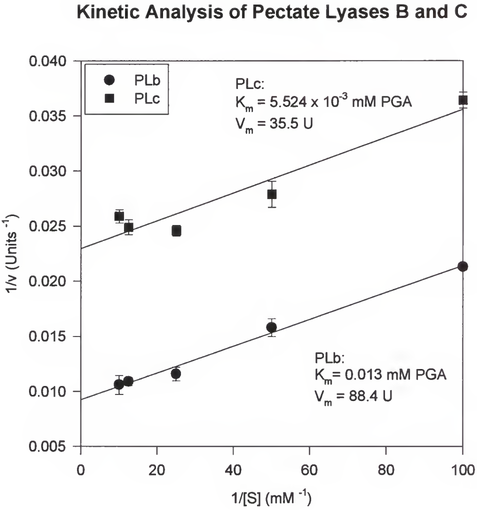


Figure 5: Double reciprocal plot for activity data from pectate lyases B and C at 0.4 mM and 0.45 mM calcium chloride, respectively. Data is from 1 ml triplicate reactions initiated with 13U of each enzyme with subsequent monitoring of the change in absorbance at 234 nm. Initial amounts of enzyme added to each reaction were based upon the amount of activity detected in non-preincubated reactions consisting of 0.1% PGA (w/v) in 50 mM Tris-HCl [pH 8.5] : 0.2 mM $CaCl_2$.

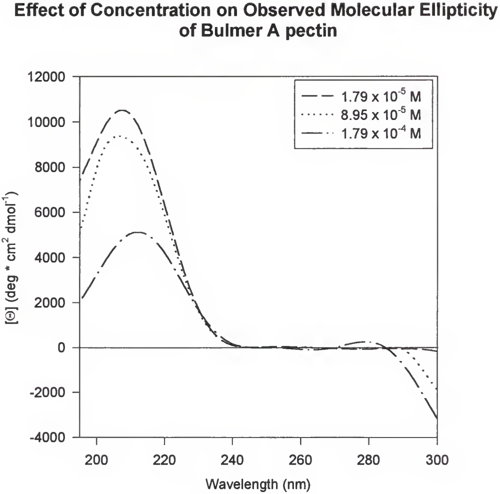


Figure 6: Changes in circular dichroism as an effect of change in concentration of Bulmer A (DM = 68). Spectra given are the average of 3 scans collected in a 1 cm cell at 25 C.

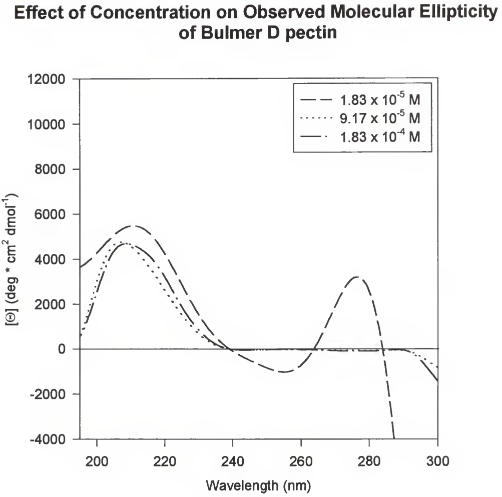


Figure 7: Changes in circular dichroism as an effect of change in concentration of Bulmer D (DM = 39) pectin. Spectra given are the average of 3 scans collected in a 1 cm cell at 25 C.

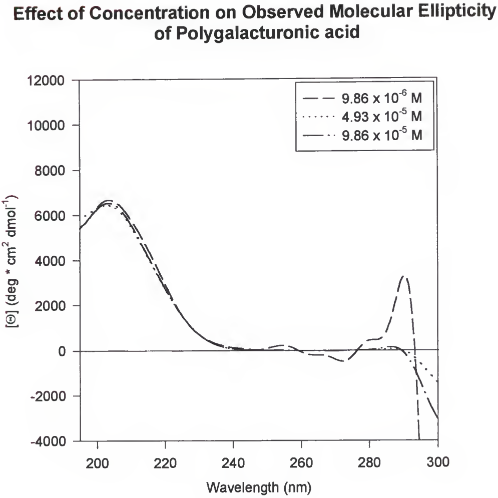


Figure 8: Changes in circular dichroism as an effect of change in concentration of PGA. Spectra given are the average of 3 scans collected in a 1 cm cell at 25 C.

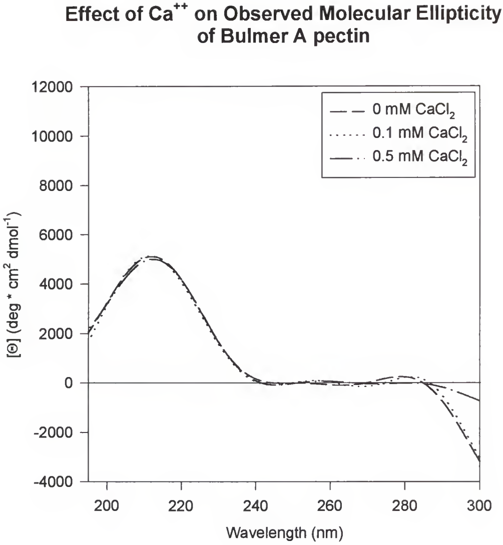


Figure 9: Changes in circular dichroism as an effect of change in concentration of Ca^{++} for Bulmer A (DM = 68) pectin. Pectin concentration was held constant at 1.79×10^{-4} M . Spectra given are the average of 3 scans collected in a 1 cm cell at 25 C.

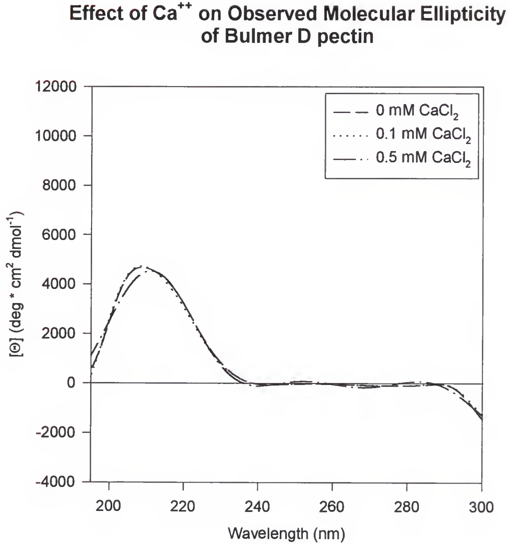


Figure 10: Changes in circular dichroism as an effect of change in concentration of Ca^{++} for Bulmer D (DM = 39) pectin. Pectin concentration was held constant at 1.83 x 10^{-4} M . Spectra given are the average of 3 scans collected in a 1 cm cell at 25 C.

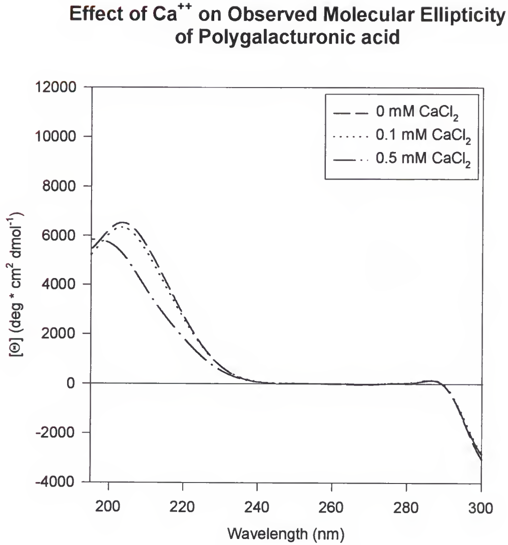


Figure 11: Changes in circular dichroism as an effect of change in concentration of Ca^{++} for polygalacturonic acid PGA concentration was held constant at 9.86×10^{-4} M. Spectra given are the average of 3 scans collected in a 1 cm cell at 25 C.

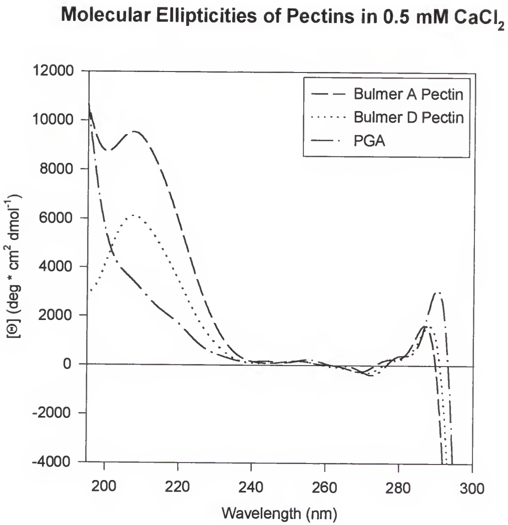


Figure 12: Observed molecular ellipticities of Bulmer A, Bulmer D and PGA after 72 hours of incubation in the presence of 0.5 mM $CaCl_2$. All polysaccharides were measured at 0.1% (w/v) and spectra represent the average of 3 scans collected in a 1 cm pathlength cell at 25 °C.

Depolymerization kinetics monitored by reversed-phase HPLC

Subtle differences in the products formed during the initial stages of the depolymerization of PGA by pectate lyases B and C can be seen in Figure 8. At all concentrations of calcium chloride tested, pectate lyase B produces much higher amounts of Δ-4,5-unsaturated oligogalacturonides of DP2-4 during the initial 4 hours of reaction. Pectate lyase B produces unsaturated DP2 in equal amounts despite the calcium chloride concentration in the reaction during the initial four hours of reaction. Four nanomoles of unsaturated DP2 are detected by 2.5 hours of reaction, and the detected levels remain constant through to 4 hours of reaction. After 24 hours of digestion of PGA by PLb however, the amounts of detected unsaturated DP2 differ sharply based upon the concentration of calcium present in the reaction mixture. Seven nanomoles of unsaturated DP2 are detected in the 0.2 mM $CaCl_2$ sample, compared to 9 and 11 nanomoles in the 0.4 mM and 1.0 mM $CaCl_2$ samples, respectively. Production of unsaturated DP3 from PGA by pectate lyase B appears to be highly dependent on the calcium ion concentration in the reaction mixture. After 30 minutes of reaction, over 10 nanomoles of unsaturated DP3 are detected in the 0.2 mM $CaCl_2$ sample, compared to just over 4 nanomoles in the 0.4 mM and 1.0 mM samples. After an hour of reaction, over 15 nanomoles of unsaturated DP3 are detected in the reaction mixture containing 0.2 mM $CaCl_2$, compared to 10 and 8 nanomoles in those containing 0.4 mM and 1.0 mM $CaCl_2$, respectively. For the remaining sampling times in the experiment, the detected amounts of unsaturated DP3 in the samples containing 0.2 mM and 0.4 mM $CaCl_2$ remain equivalent (18 nanomoles). This is not the case in the reaction mixtures containing 1.0 mM $CaCl_2$, where the detected amounts of unsaturated DP3 remain much lower (12 nanomoles) until 24 hours after reaction initiation when 15 to 17 nanomoles of unsaturated trimer is detected in all of the samples.

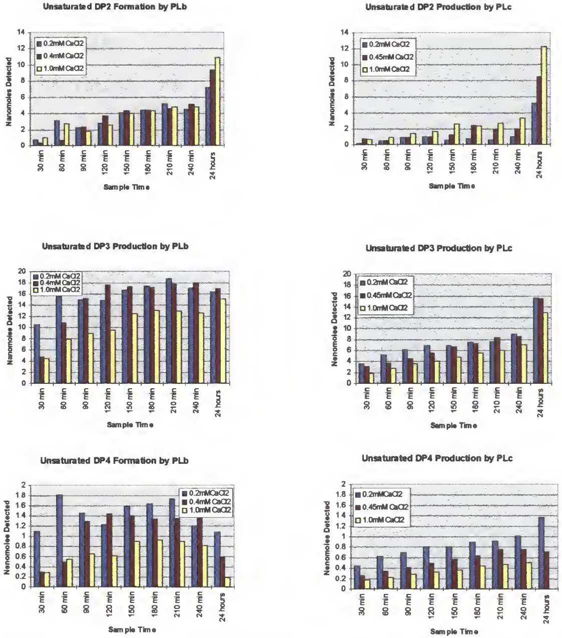


Figure 13: Quantitation of unsaturated oligogalacturonides by reversed phase HPLC with 234 nm detection. Data is from 50 ul injections taken from 4 ml reactions in which substrate (PGA) was allowed to equilibrate in the presence of 0.2 mM, 0.4 mM (PLb reactions), 0.45 mM (PLc reactions) and 1.0 mM $CaCl_2$.

Unsaturated DP4 production by pectate lyase B also appears to be highly dependent on the calcium ion concentration present in the reaction mixture. After 30 minutes of digestion, over 4 times as many tetrameric oligogalacturonides are present in the reaction mixtures containing 0.2 mM $CaCl_2$ as are present in those containing 0.4 mM and 1.0 mM $CaCl_2$. This trend continues until 90 minutes of reaction, when the unsaturated DP4 amounts in the 0.2 mM and 0.4 mM $CaCl_2$ reactions are equivalent at 1.3-1.4 nanomoles. At all concentrations of $CaCl_2$, the amounts of unsaturated DP4 present in the reaction mixtures increases through the initial 3.5 hours of the reaction, and then decreases during the remaining 20.5 hours of the study.

Pectate lyase C continuously produces unsaturated DP2 at all $CaCl_2$ concentrations during the sampling times tested. At increasing incubation times, the amount of unsaturated DP2 detected in each sample is directly dependent on the $CaCl_2$ concentration of the reaction mixture. After 4 hours of reaction, 3 nanomoles of unsaturated DP2 are detected in the sample containing 1.0 mM $CaCl_2$, compared to 1 and 2 nanomoles in those containing 0.2 mM and 0.45 mM $CaCl_2$, respectively. After 24 hours of reaction, this difference in detected dimeric oligogalacturonides is still apparent with 5, 8.5, and 12 nanomoles of unsaturated DP2 detected in the reaction mixtures containing 0.2 mM, 0.45 mM and 1.0 mM $CaCl_2$. Unsaturated DP3 production is constant in all the reaction mixtures as well, with a constant increase in detected DP3 observed for all samples. During the initial 2 hours of digestion, more unsaturated DP3 is detected in the 0.2 mM $CaCl_2$ reaction mixture (3, 5, 6 and 7 nanomoles of unsaturated DP3 after 30, 60, 90 and 120 minutes of reaction, respectively) than in the reaction mixtures containing higher concentrations of $CaCl_2$. Equivalent amounts of detected trimeric oligogalacturonides are present in the 0.2 mM and 0.45 mM $CaCl_2$ reaction mixtures from 3.5 hours to the completion of the study at 24 hours. When added to reaction mixtures containing 0.2 mM $CaCl_2$, pectate lyase C produced constantly increasing amounts of unsaturated DP4, culminating in 1.35 nanomoles of detected tetrameric oligogalacturonides after 24 hours of reaction. Reactions run in the presence 0.45 mM and 1.0 mM $CaCl_2$ show a similar increase in detected unsaturated DP4 amounts until 4 hours of reaction, where the amount of DP4 detected levels off. From 4 to 24 hours, all of the DP4 produced during the initial stages of depolymerization is consumed in the 1.0 mM $CaCl_2$ reaction, whereas only 0.05 nanomoles have disappeared from the reaction mixture containing 0.45 mM $CaCl_2$.

Activities of pectate lyases B and C towards pectin substrates with varying degrees of methylation

As seen in Figure 14, pectate lyase B possesses a higher initial activity towards Bulmer A pectin than either of the other substrate types when the substrates are incubated in the presence of 0.2 mM $CaCl_2$. In fact, the enzyme exhibits the same activity (17 units) towards this substrate, regardless of the calcium ion concentration present in the reaction mixture. When Bulmer D pectin is the substrate, PLb shows an activity maximum of 18.5 units at 0.4 mM calcium chloride. PLb displays maximum activity at 0.4 mM $CaCl_2$, with 13.4 units detected in the sample, which is 176% higher than the activity demonstrated when PGA is incubated in the presence of 0.2 mM $CaCl_2$. There is no significant change in activity when PGA is incubated in the presence of 1.0 mM $CaCl_2$ relative to that seen at 0.4 mM $CaCl_2$.

At all calcium ion concentrations tested, PLc shows the highest activity towards Bulmer A and D pectins, with the initial activity towards Bulmer A differing most (10%) when the substrates are incubated in the presence of 1.0 mM $CaCl_2$ (Figure 15). As was seen in the PLb samples, PGA is a better apparent substrate than Bulmer D pectin, with the

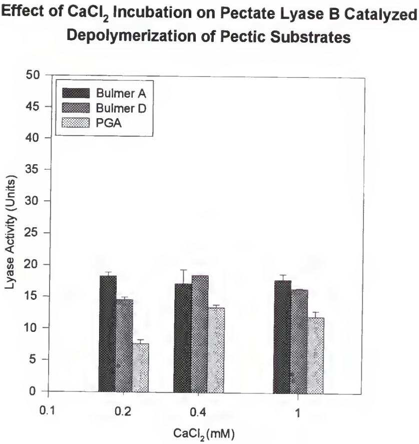
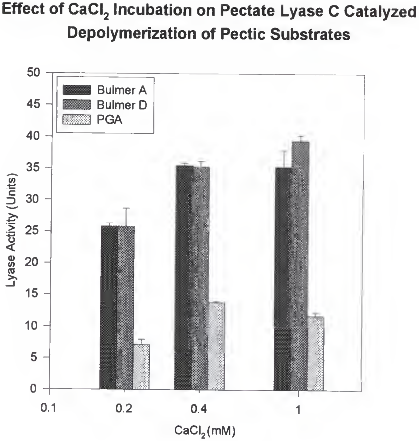
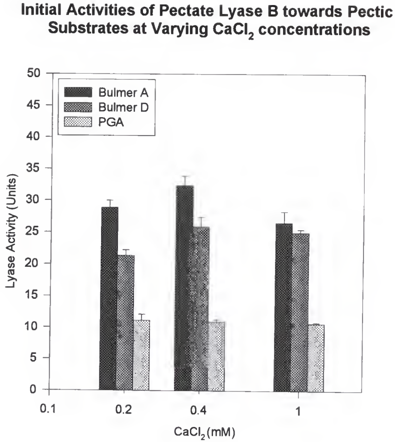

Effect of $CaCl_2$ Incubation on Pectate Lyase B Catalyzed Depolymerization of Pectic Substrates

Figure 14: Initial activities of PLb with respect to pectic substrates at varying $CaCl_2$ concentrations. All substrates were incubated for 72 hours in the presence of 0.2 mM, 0.4 mM or 1.0 mM $CaCl_2$ prior to the initiation of the reaction. Activities shown are from 1.0 ml triplicate reaction mixtures with enzymatic activity determined by the change in absorbance at 234 nm.



Effect of $CaCl_2$ Incubation on Pectate Lyase C Catalyzed Depolymerization of Pectic Substrates

Figure 15: Initial activities of PLc with respect to pectic substrates at varying $CaCl_2$ concentrations. All substrates were incubated for 72 hours in the presence of 0.2 mM, 0.4 mM or 1.0 mM $CaCl_2$ prior to the initiation of the reaction. Activities shown are from 1.0 ml triplicate reaction mixtures with enzymatic activity determined by the change in absorbance at 234 nm.



Initial Activities of Pectate Lyase B towards Pectic Substrates at Varying $CaCl_2$ concentrations

Figure 16: Initial activities of PLc with respect to pectic substrates at varying $CaCl_2$ concentrations. All substrates were incubated for 72 hours in the presence of 0.2 mM, 0.4 mM or 1.0 mM $CaCl_2$ prior to the initiation of the reaction. Activities shown are from 1.0 ml triplicate reaction mixtures with enzymatic activity determined by the change in absorbance at 234 nm.

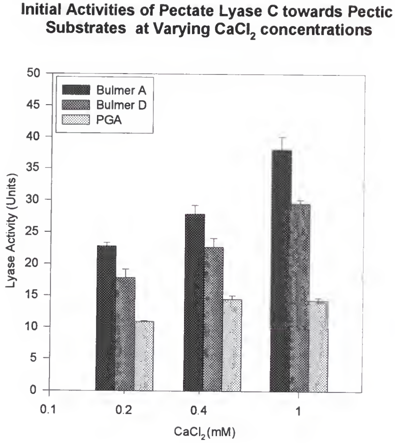


Figure 17: Initial activities of PLc with respect to pectic substrates at varying $CaCl_2$ concentrations (0.2 mM, 0.45 mM, 1.0 mM). Activities shown are from 1.0 ml triplicate reaction mixtures with enzymatic activity determined by the change in solution absorbance at 234 nm.

highest activity (13.7 units) seen at 0.45 mM CaCl2. The initial activity of the enzyme does decrease as the calcium ion concentration is increased from 0.45 mM to 1.0 mM, dropping 15% to 11.6 units at the highest calcium concentration.

Figure 16 shows the initial activities of pectate lyase B towards the pectic substrates at varying calcium chloride concentration without pre-incubation of substrate and calcium ions. When Bulmer A pectin is presented to PLb as substrate, the enzyme shows maximum activity when $CaCl_2$ is added to the reaction mixture (25.9 units). For both Bulmer A and D pectins, PLb shows an increase in activity when the $CaCl_2$ concentration is increased from 0.2 mM to 0.4 mM and then a decrease in activity as the $CaCl_2$ concentration is raised again to 1.0 mM. PLb shows a constant activity of 10.5 units with PGA as substrate, regardless of the $CaCl_2$ concentration in the reaction mixture.

Unlike PLb, pectate lyase C displays quite different behavior when the substrate and calcium ions are not pre-incubated prior to addition of enzyme, as seen in Figure 17. For all of the Bulmer pectins, the apparent activity of PLc increases as the calcium ion concentration is increased. As the $CaCl_2$ concentrations are increased from 0.2 mM to 1.0 mM, the activity of PLc towards the Bulmer A or D pectins is increased 67% for each of the substrates. When PGA is used as the substrate however, the apparent activity of PLc towards the polymer increases to a maximum of 14.5 units at 0.45 mM $CaCl_2$ and then decreases to 14.3 units as the calcium ion concentration is raised further to 1.0 mM.

Measurement of extent of conversion of pectin substrates by pectate lyases B and C

As seen in Table 2, when Bulmer pectins are presented to PLb and PLc as substrate, the reaction has not reached limit within 24 hours. The pectate lyase C catalyzed reactions display a higher absorbance at 234 nm than the PLb reactions after 24 hours with Bulmer

Table 2: Reaction progress and lyase activity remaining reaction mixtures containing pectic substrates after 48 hours of digestion by pectate lyase B or C

Enzyme	Substrate	[Ca^{++}]	Substrate/ Ca^{++} incubation time (hours)	Absorbance (234nm) after 24 hours	Absorbance (234nm) after 48 hours	Lyase Activity remaining (% control)
PLb	BulA	0.2 mM	0	1.48 ± 0.02	2.32 ± 0.06	109
PLb	BulD	0.2 mM	0	1.44 ± 0.02	3.28 ± 0.03	102
PLc	BulA	0.2 mM	0	2.14 ± 0.03	2.29 ± 0.01	111
PLc	BulD	0.2 mM	0	2.00 ± 0.07	3.02 ± 0.13	91
PLb	PGA	0.2 mM	0	3.48 ± 0.04	3.44 ± 0.09	101
PLb	PGA	0.2 mM	72	3.39 ± 0.01	3.42 ± 0.03	82
PLc	PGA	0.2 mM	0	3.49 ± 0.01	3.58 ± 0.02	81
PLc	PGA	0.2 mM	72	3.44 ± 0.08	3.41 ± 0.05	74
PLb	PGA	1.0 mM	0	3.48 ± 0.06	3.64 ± 0.02	130
PLb	PGA	1.0 mM	72	3.32 ± 0.006	3.47 ± 0.01	126
PLc	PGA	1.0 mM	0	3.57 ± 0.007	3.69 ± 0.04	110
PLc	PGA	1.0 mM	72	3.41 ± 0.005	3.52 ± 0.02	146

Table 3: Δ-4,5-unsaturated oligogalacturonides of DP 2-4 detected by reversed-phase HPLC in 1 ml reaction mixtures after 48 hours of digestion with pectate lyases B or C.

Enzyme	Substrate	[Ca^{++}]	Substrate/Ca^{++} incubation time (hours)	μg ΔDP2 detected	μg ΔDP3 detected	μg ΔDP4 detected	% Conv. after 48 hr
PLb	BulA	0.2 mM	0	38	80	16	13.4
PLb	BulD	0.2 mM	0	62	94	40	19.4
PLc	BulA	0.2 mM	0	40	88	24	15.1
PLc	BulD	0.2 mM	0	52	84	42	17.8
PLb	PGA	0.2 mM	0	102	306	0	41.0
PLb	PGA	0.2 mM	72	90	298	0	38.7
PLc	PGA	0.2 mM	0	112	294	0	40.6
PLc	PGA	0.2 mM	72	94	286	0	38.0
PLb	PGA	1.0 mM	0	152	196	0	34.9
PLb	PGA	1.0 mM	72	148	186	0	33.4
PLc	PGA	1.0 mM	0	220	78	0	29.9
PLc	PGA	1.0 mM	72	218	64	0	28.1

Amounts shown are the average of 2 independent runs made under the chromatographic conditions previously described. % Conversion values are based upon 1 mg of substrate at reaction initiation.

pectins as substrate, suggesting a further extent of reaction, but after 48 hours of digestion, no significant differences exist between the two enzymes. When PGA is presented to the enzymes as a substrate, regardless of the calcium concentration tested, all reactions have reached limit by 24 hours as evidenced by the lack of an absorbance increase in any of the PGA samples after 48 hours of digestion. The enzyme is active in all cases, possessing at least 74% of the control activity after 48 hours, despite being incubated at room temperature during the time course of the study.

Both enzymes produce Δ-4,5-unsaturated tri-galacturonides as the limit product with Bulmer A as substrate (80 μg for PLb and 86 μg for PLc) with low amounts of tetramer (16 μg and 24 μg for PLb and PLc, respectively) and moderate amounts of dimer (38 μg and 40 μg for PLb and PLc) detected. Given the amount of substrate present at the initiation of the reaction (1 mg), the extent of conversion for both enzymes with Bulmer A as a substrate are comparable with pectate lyase B depolymerizing 13% of the initial substrate and pectate lyase C depolymerizing 15%. The similarities in product formation are also seen when Bulmer D is the starting substrate. In reactions catalyzed by pectate lyase B, 62 μg of dimer, 94 μg of trimer and 40 μg of tetramer are detected, compared to 52 μg of dimer, 84 μg of trimer and 42 μg of tetramer detected in reaction mixtures catalyzed by pectate lyase C. Pectate lyase B depolymerizes 19% of the initial Bulmer D, whereas pectate lyase C depolymerizes 18%. PGA appears to be depolymerized equally well by both lyases when the calcium concentration is 0.2 mM, and pre-incubation seems to have a slight effect on the extent of conversion for both enzymes. With no pre-incubation, pectate lyase B produces 306 μg of detectable trimer and 102 μg of detectable dimer after 48 hours of reaction with 1000 μg/ml PGA in 0.2 mM $CaCl_2$, and pectate lyase C produces 294 μg of trimer and 112 μg of dimer under the same conditions. When the substrate is pre-incubated in the presence of 0.2 mM $CaCl_2$, the amounts of detectable dimer and trimer in the PLb reaction drop to 90 μg and 298 μg while pectate lyase C catalyzed reactions have 286 μg and 94 μg of trimer and dimer detected, respectively.

The decrease in detected products when PGA is pre-incubated is also seen in the extent of conversions observed, with pectate lyase B depolymerizing 38% of the PGA if it is incubated in 0.2 mM $CaCl_2$ and 41% if not pre-incubated, while pectate lyase C depolymerizes 38% of the substrate if it is pre-incubated, and 41% without pre-incubation. The most pronounced difference seen between the enzymes occurs when each lyase is presented with PGA as substrate in the presence of 1.0 mM $CaCl_2$. Under these conditions, 152 μg of dimer and 196 μg of trimer are detected in the non-pre-incubated reaction catalyzed by pectate lyase B, compared to 220 μg of dimer and 78 μg of trimer detected in pectate lyase C catalyzed reactions. When PGA, incubated in the presence of 1.0 mM $CaCl_2$, is presented to pectate lyase B as a substrate, 148 μg of dimer and 186 μg of trimer are produced while pectate lyase C produces 218 μg of dimer and 64 μg of trimer. Neither enzyme depolymerizes as much PGA when the calcium chloride concentration is 1.0 mM, with PLb depolymerizing 33% and 35% with and without pre-incubation, compared to 28% and 30% for pectate lyase C under the same conditions.

Discussion

Optimization of calcium ion concentrations for the lyase catalyzed depolymerization reaction

The affinities of each lyase for calcium ions can be seen to differ slightly in Figure 4. Pectate lyase B has a higher affinity (0.4 mM) for calcium than pectate lyase C (0.45 mM), which differs from previously observed experiments using the same substrate (Hurlbert, 1996) in which PLc was shown to have a higher affinity for calcium than PLb. This difference could be due to the procedural change introduced in the experiments described in this work involving preincubation of substrate in the presence of each calcium chloride solution in buffer. The standard deviation of each triplicate set is greatly reduced when the substrate and calcium ions are incubated together prior to addition of lyase, allowing a much more accurate determination of the optimum calcium concentration for each enzyme. The absolute requirement of each enzyme for calcium has been previously stated, as has the chelative nature of the substrate with respect to the cation. By allowing incubation of the substrate and calcium, calcium-mediated cross-bridged dimers of polysaccharide molecules may be forming that more closely resemble natural substrate structures. The previously published calcium concentration optima may have provided a better idea of the ability of each lyase to compete with the substrate for solution-accessible calcium ions, while the experiments outlined in this work are providing information regarding the ability of each enzyme to degrade substrate when it has adopted stable solution structures. In the latter situation, the lower soluble calcium ion requirement observed for pectate lyase B would favor initial attacks on plant tissue *in vivo* where calcium ions may be sequestered by the structural polymers comprising the cell wall of the host. Both enzymes lack the ability to depolymerize large aggregates of polygalacturonic acid molecules as supported by the sharp decrease in the observed enzymatic activity at calcium concentrations higher than the optimum for each lyase.

Kinetic comparisons of pectate lyases B and C

Pectate lyase B shows a linear double reciprocal plot with a derived K_m of 0.013 mM PGA. Based upon the shape of the curve and the previously published depolymerization profile for PLb (Preston et al. 1992), it appears that at 0.4 mM $CaCl_2$, the enzyme has no difficulty making endolytic cleavages of the high DP substrate at any of the concentrations of PGA studied. This does not appear to be the case for the reactions involving pectate lyase C at its optimum calcium concentration (0.45 mM). A reliable K_m value for PLc cannot be obtained due to the decrease in enzymatic activity at substrate concentrations higher than 0.04 mM. The extrapolated K_m value of 5.5×10^{-3} mM may not be accurate due to this observed decrease in enzymatic activity. This activity decrease could be attributed to a lower activity of PLc towards oligogalacturonides with DP's lower than those of the initial substrate. In other words, after the enzyme has made several endolytic cleavages of the longer starting polymeric substrate, it does not depolymerize the cleaved fragments as efficiently and hence the activity decrease. If this were the case however, the activity decrease after initial cleavages of the high DP substrate would be reflected in a decrease in the slope of the raw time course data (change in absorbance at 234 nm versus time), something that is not observed. Therefore, it is more likely, based upon the CD data presented in Figures 6 and 7 and discussed below, that pectate lyase C has difficulty in binding and/or cleaving the high DP substrate molecules once they have dimerized and aggregated. The dimerization events should occur at even low substrate concentrations, but, as the name implies, the aggregation effect is substrate concentration dependent, and hence the lower observed initial activity at higher substrate concentrations.

Effects of calcium ions and substrate concentration on solution structure of substrate molecules

PGA has been shown to tightly bind calcium ions in solution and in so doing, form dimers of polysaccharide molecules (Morris et al., 1982). In Figures 6-8, it appears that other effects, possibly resulting from hydrophobic interactions, also serve to influence the structures of pectin molecules, as evidenced by the decrease in molecular ellipticity. These structural changes may reflect the formation of duplexes of PGA molecules or larger aggregates of duplexes (Walter and Matias, 1991). As the pectin concentration increases in the absence of calcium ions, the extent of dimer formation is inversely proportional to the degree of methylation. This type of interaction is not seen in polygalacturonic acid, which possesses no methyl esters attached to the carboxyl carbon of the uronate residues, seen to some extent in partially methylated Bulmer D pectin, and is very strong in the highly methylated Bulmer A pectin. When calcium ions are introduced into the system, two additional effects are seen (Figure 7). In the Bulmer D pectin samples, the spectra of the samples at each different concentration of pectin begins to separate, with dimer formation seen in the Bulmer D sample as the $CaCl_2$ concentration is increased to 0.5 mM. This behavior is also seen in the PGA sample at a much more attenuated level. Calcium binding requires at least 14 sequential uronate residues in the polymer (Rees, 1982), so it follows that the effect should be more pronounced as the DM of each polysaccharide decreases. The second effect seen in all pectic samples was the appearance of a molecular ellipticity maximum at 285 nm and a minimum at 265-270 nm. These peaks only appear at the lowest concentration of each pectin studied, with PGA showing the highest maximum (3105 deg·cm^2·decimole^{-1}) and Bulmer A and D pectins showing similar, yet smaller maxima (1631 and 1609 deg·cm^2·decimole^{-1}, respectively). The structural features contributing to these peaks are unknown and previously uncharacterized in the literature.

Depolymerization kinetics monitored by reversed-phase HPLC

An examination of Figure 13 serves to magnify the apparent differences between pectate lyases B and C with respect to their abilities to depolymerize calcium-cross-bridged substrate. Previous kinetic HPLC analysis of both enzymes using much smaller DP substrate in the absence of pre-incubation with Ca^{++} (Preston et al., 1992) showed strong similarity in product formation between these enzymes. When a higher DP substrate is used (2x higher than the previous studies) and is allowed to form Ca^{++}-complexed duplex structures of polymer molecules in solution, the apparent differences in the endolytic abilities of PLb and PLc are highlighted. Despite the fact that both reactions were set up with the same amount of enzyme (based upon reaction mixtures run at 0.2 mM $CaCl_2$: 0.1% PGA, without pre-incubation) pectate lyase B generates much more of each oligogalacturonide at all calcium concentrations studied. This supports the concept of pectate lyase B being more effective at endolytic cleavages than pectate lyase C as put forth previously (Hurlbert, 1996). Pectate lyase C consistently produces all of the oligogalacturonide DP's detected, suggesting that it has a much more difficult time making initial cleavages of PGA dimers. However, given time, enough endolytic cleavages are made by PLc in the high DP substrate molecules that the dimer structure is disrupted (by taking the average DP of the polymer molecules to less than 14), and the limit product amounts are equivalent to those seen in the pectate lyase B reactions.

An interesting observation for both enzymes is the production of dimeric oligogalacturonides after 24 hours of incubation in the 1.0 mM $CaCl_2$ reaction. More of these molecules accumulate in these reaction mixtures than in those of either of the other calcium concentrations studies, suggesting that in addition to endolytic cleavages of DP 57 PGA that produce two products with DP > 30, the enzymes are also "nicking" the high DP molecules at a consistent rate. In the 1.0 mM $CaCl_2$ sample with PLc added to it, after 24 hours of digestion, the amount of detected Δ-4,5-unsaturated digalacturonic acid is equivalent to that of trigalacturonic acid, something not seen in the PLb reaction under the same conditions, further supporting the theory that PLc may not be as effective in degrading aggregated PGA molecules.

Activities of pectate lyases B and C towards pectin substrates with varying degrees of methylation

The initial activities of each lyase towards methyl esterified substrates provides key information with respect to their abilities to bind and subsequently cleave substrate. Pectate lyase B shows similar activity towards Bulmer pectins A and D, regardless of the calcium concentration in which the substrate was incubated. This suggests that any effects of calcium ions on the structure of substrate molecules in solution are negligible and that the most important factor in the recognition and cleavage of these substrates relative to polygalacturonic acid is the presence of the methyl ester attached to the carboxyl groups of each monomeric uronic acid. The higher activity of PLb towards these substrates relative to PGA, which is expected to form duplexes of high DP molecules, suggests two possibilities. In the first scenario, either the hydrophobic interactions that may cause limited dimerization of the substrate do not result in structures that are as inaccessible to PLb as the calcium cross-bridged PGA molecules and are therefore more easily cleaved by the enzyme. The second, and most probable explanation, is that the stretches of non-methyl esterified uronic acids present in Bulmer A and D are not cross-bridged to other polymer molecules through calcium ions, and are therefore much more readily accessible to lyase cleavage than is PGA. This explanation makes sense given the number of sequential, ionized carboxyl groups necessary to form the cross bridged structures (14 residues) and the extent of conversion data given in Tables 2 and 3. This is not to suggest that the initial dimerization events driven by hydrophobic interactions in Bulmer A and D pectins are irrelevant to the depolymerization process as far as PLb is concerned, as Figure 16 shows. When PLb is introduced into the reaction before a substantial number of these hydrophobic interactions have occurred, over twice the initial activity is observed on those substrates. The much lower activity displayed by PLb towards PGA in the absence of pre-incubation with $CaCl_2$, relative to Bulmer A and D pectins, is much more difficult to explain. It may be that the formation of the calcium-cross-bridged dimers is very rapid, with the calcium chelation events occurring upon addition of calcium ions to solutions of substrate, causing the formation of substrate structures that require breakdown to much smaller DP molecules which can then be further processed, a phenomenon that has been previously reported for PLb based upon time course of activity data (Hurlbert, 1996).

The initial activity towards pectic substrates for pectate lyase C is similar to pectate lyase B when the substrates are pre-incubated in the presence of calcium ions. Bulmer A and D pectins appear to be equivalent substrates, yielding activities of 25, 35 and 37 units at 0.2 mM, 0.45 mM and 1.0 mM $CaCl_2$, respectively. These activities are much higher than those observed for PGA (7, 14, and 12 units at 0.2 mM, 0.45 mM and 1.0 mM $CaCl_2$, respectively). This suggests that PLc can bind and cleave methylated substrates much more effectively than PLb. In the absence of pre-incubation, a slight decrease in initial activity is seen in the Bulmer A samples at 0.2 mM and 0.45 mM $CaCl_2$ concentrations, and no significant change is seen at 1.0 mM $CaCl_2$. This is not the case with Bulmer D pectin, which shows a lowered activity under all calcium ion concentrations studied in the absence of pre-incubation. No explanation can be provided for this observation. As expected, in the absence of pre-incubation of substrate and calcium ions, PLc shows a much higher initial activity towards PGA relative to the activities observed for the enzyme with the pre-incubated samples of these substrates.

Measurement of extent of conversion of pectin substrates by pectate lyases B and C

Neither lyase can be seen to depolymerize Bulmer A or D pectin very effectively. The initial activity differences seen between PLb and PLc with respect to these pectins are not reflected in the total extent of conversion determined at limit. This suggests that PLc can more readily bind and cleave partially methylated stretches of the polymers than PLb, but neither enzyme can effectively degrade these molecules, hence the evolution and retention of pectin lyase and pectin hydrolase by *Erwinia* species. Both PLb and PLc depolymerize PGA pre-incubated in the presence of 0.2 mM $CaCl_2$ to the same extent, as was seen in the kinetic HPLC study, with the same amounts of limit products produced by each enzyme. The overall extent of conversion of PGA pre-incubated in the presence of 1.0 mM $CaCl_2$ differs between PLb and PLc, confirming that PLc does not bind and cleave the calcium cross-bridged PGA dimers as effectively as does PLb.

CHAPTER 3
STRUCTURAL PROPERTIES OF PECTATE LYASES DETERMINED BY CIRCULAR DICHROISM

Introduction

Circular Dichroism (CD) is an optical phenomenon resulting from the differential absorption of left-handed circularly polarized light and right-handed circularly polarized light (Mulkerrin, 1996), and occurs only in regions of the spectrum where the sample absorbs (Tinoco et al., 1985). Because of this, the primary wavelength range of interest with respect to protein CD is the ultraviolet region of the electromagnetic spectrum. In this region, the primary chromophores are amide groups, aromatic amino acids and the disulfide bond. The observed CD for the aromatic amino acids and disulfide bonds occurs in the near-UV region of the spectrum and can provide information about the specific environment surrounding each chromophore. For this reason, some CD studies focus on the 250 nm to 300 nm range, following the changes in observed CD as a means of following the structural changes occurring in a protein sample in response to experimentally provided stimuli. By far the most prevalent chromophore in a protein is the amide chromophore however, and the most useful structural information regarding a particular sample can be found in the far-UV region where amide groups show absorption maxima. In a polypeptide or protein, the amide chromophore is not isolated, but rather arranged in a continuous progression throughout the length of the polymer. The amide group shows two electronic transitions when it absorbs incident energy, an $n\pi^*$ transition and a $\pi\pi^*$ transition, and the wavelengths at which these transitions are manifest is an accurate indicator of the three dimensional arrangement of the amide chromophores (Mulkerrin, 1996). In a folded protein, there are discreet units of these three dimensional arrangements, known as secondary structural elements (α-helix, β-structures and random coil). Using model polypeptides that fold into a single secondary structure type, the electronic transitions for amide chromophores arranged in each of the three types of secondary structure have been identified and serve as a basis for characterizing other proteins. For the three secondary structural elements described, the electronic transitions (with positive or negative ellipticity) can be found at 208 nm (negative band) and 222 nm (negative band) for α-helices; 198 nm (positive band) and 215 nm (negative band) for β-structures; and 195 nm (positive band) and 217 nm (negative band) for random coil or unordered proteins (Woody, 1996).

In 1995, the CD spectra of pectate lyases C and E were published (Sieber et al., 1995), representing the first published spectra of β-helical proteins. The structure of each protein had been previously determined using X-ray crystallography (Yoder et al., 1993) and were found to be similar, consisting of approximately 25% parallel β-sheet (26.6 % for PLc and 24.5% for PLe). This observation was reflected in the similarity of the CD spectra obtained for the proteins. No change was observed in the CD spectrum for either protein when 1 mM $CaCl_2$ was added to the sample buffer, suggesting that the binding of calcium by the lyases does not result in a significant conformational change in the proteins.

One possible use of CD spectra is to determine the individual structural elements present in a protein and their relative percentage (Venyaminov and Yang, 1996; Mulkerrin, 1996). To accomplish this, a variety of algorithms have been developed, all of which rely upon sets of reference proteins of known secondary and tertiary structure. Unfortunately, for β-sheets, there is a much larger degree of structural variation (sheet twisting, parallel and anti-parallel sheets, variations in β-turns) possible than there is for α-helices and those variations have a large effect on the observed CD spectrum for a protein consisting of β-sheets. Because of this, attempts to deconvolute the spectra of pectate lyase C and E did not meet with success (Sieber et al., 1995). Future reference protein sets could be enhanced by the addition of spectra of known proteins containing large amounts of β-sheets in a well-defined arrangement. The addition of the CD spectrum of pectate lyase B to the collection of β-helical protein spectra already obtained could be an important addition to this reference set.

Circular dichroism also provides an excellent opportunity to measure the effects of denaturing conditions on the structure of proteins. No work has been done examining these effects on β-helical proteins, and much of the work in this chapter addresses this deficit. Based upon the X-ray crystallographic structures of PLs c and e, the folding motif possessed by the pectate lyases should be highly stable. The helical core that dominates overall fold of the lyases is composed of 3 parallel β-sheets, which, in turn, are composed of 6-10 β strands. This arrangement should allow for a large number of intrastrand hydrogen bonds (Kyte, 1995), helping to maintain the structure. In addition, there are several side chain stacks present in the core of the molecule, including aliphatic (6 stacks in PLc and 4 in PLe, each consisting of at least 4 aliphatic amino acid side chains), asparagine (6 residues in PLc, 3 in PLe), serine (3 residues in PLc, 2 in PLe), and aromatic side chains (3 in PLc and 4 in PLe) which should help to stabilize the hydrophobic core of the lyases. Monitoring the changes in molecular ellipticity (CD) and lyase activity will allow for a cross comparison of the two families of lyases, as well as examine the structural differences that have evolved between two homologous enzymes.

Materials and Methods

Guanidine hydrochloride denaturation of pectate lyases B, C and E

Protein samples were purified and characterized as described previously. A stock solution of 8 M guanidine in deionized water was prepared by heating 8 ml of water to 40 °C and slowly adding 15.28 g of guanidine hydrochloride (GdnHCl, Sigma Cat # G-7153, >99.5% pure) with constant stirring. The resulting solution was filtered through a 0.45 μm membrane filter (Millipore GV) with the absorbance of the solution checked before and after filtration. Using λ_{max} at 206 nm, the absorbance before filtration was 2.87 and 2.86 after filtration with no change in the spectrum from 190 nm to 820 nm evident. This stock solution was degassed by sonication under vacuum and dispensed into 1.5 ml microcentrifuge tubes such that the final concentration in 500 μl would be: 0 M, 0.25 M, 0.5 M, 0.75 M, 1.0 M, 1.5 M, 2.0 M, 3.0 M, 4.0 M, 5.0 M, and 6.0 M. Deionized, degassed GVWP filtered water was added to the GdnHCl aliquots to a volume of 480 μl. Protein solutions were made from stocks to concentrations of 125 μM in degassed, deionized GVWP filtered water and 20 μl of these solutions were added to each microcentrifuge tube. Each tube was mixed and allowed to sit at room temperature for 3 hours to allow for denaturation to reach equilibrium. Upon completion of the incubation period, 50 μl was removed from each denaturation mixture and stored in a microcentrifuge tube at room temperature for activity analysis.

Circular dichroism measurements of guanidine hydrochloride denatured pectate lyase solutions

CD spectra of denatured samples of pectate lyases B, C and E were measured on a Jasco J-500C circular dichroism spectropolarimeter. Spectra were obtained from 225 nm to

195 nm at 25 °C in a 1 mm water jacketed quartz cell (Jasco, Inc. Cat # 0576) with the instrument set to a 1.0 nm bandwidth, 50 mdeg sensitivity, 0.2 nm resolution, 2 sec response, with 4 accumulations acquired per sample at a 20 nm/minute scan rate. The instrument was calibrated with (+)-10-camphorsulfonic acid (Hennessey and Johnson, 1982) prior to obtaining spectra. Before initiating any sample measurements, the baseline spectrum of GdnHCl in deionized, degassed GVWP filtered water at each concentration to be analyzed was obtained and stored to correct the sample spectra. The average spectra for all 4 accumulations were subjected to noise reduction using the Jasco J-700 for Windows Standard Analysis software (ver. 1.50.01. Jasco, Inc. 1997) (Figure 18). Upon completion of the CD measurements, 10 μl of each denaturation solution was removed and added to 990 μl of 50 mM Tris-HCl [pH 8.5] : 0.2 mM $CaCl_2$: 0.1 % (w/v) PGA in a quartz cuvette. The resulting solution was mixed well and the absorbance change at 234 nm was monitored on a Hewlett-Packard 8451A diode array spectrophotometer. Activity data is presented in Figure 19, with the units displayed standardized to the number of nanomoles of product formed per minute per milliliter of enzyme solution added to the reaction mixture.

Circular dichroism measurements of pectate lyase solutions at varying temperature

For measurement of the circular dichroism spectra of pectate lyases B, C and E in response to variable temperatures, 5 μM solutions of each lyase in 10 mM sodium phosphate pH 8.0 buffer were prepared from the purified stocks described previously. The spectrum of each sample was measured from 195 nm to 250 nm in five degree intervals from 25 °C to 70 °C. Temperature was controlled using a refrigerated/heated circulating water bath (Neslab RTE-9). Sample measurement was performed in a 1 mm pathlength, water-jacketed quartz cell (170 μl volume) connected to the water bath. After allowing 5 minutes for the sample to equilibrate at the new temperature, CD spectra were obtained using the same instrument and parameters described in the guanidine hydrochloride denaturation experiments. Baseline correction for each spectrum prior to accumulation averaging was made using the spectrum of 10 mM sodium phosphate pH 8.0 buffer. Noise reduction of the averaged spectra was performed using the Jasco J-700 for Windows Standard Analysis software (ver. 1.50.01. Jasco, Inc. 1997) (Figure 20).

Enzymatic activity measurements of pectate lyase solutions at varying temperature

Solutions of 0.5 μM pectate lyase (B, C or E) in 10 mM sodium phosphate buffer pH 8.0 were incubated for 10 minutes in a water bath (Precision, Inc.) at 30 °C. Aliquots (10 μl) of these solutions were added to 990 μl of 10 mM sodium phosphate buffer [pH 8.0] : 0.2 mM $CaCl_2$: 0.1 % PGA (w/v) in a 1 cm pathlength quartz cell, with the resulting change in absorbance at 234 nm monitored on a Hewlett-Packard 8451A diode array spectrophotometer. The temperature of the quartz cell was held constant at 30 °C by connecting the aluminum alloy cell holder to a circulating water bath (Haake, Inc.) set to 30 °C. The activity measurements for each lyase were made in triplicate. Upon completion of the activity measurements at 30 °C, the temperatures of the water baths were increased 5 °C allowed to equilibrate at the new temperature for 10 minutes and the activities measured again. This was repeated to the final measured temperature of 70°C. Data are found in Figure 21.

Results

Circular dichroism measurements of guanidine hydrochloride denatured pectate lyase solutions

Slight differences exist between the native CD spectra of each pectate lyase (Figure 18), as was seen in previous experiments studying pectate lyases C and E (Sieber et al., 1995). The CD spectrum of PLb matches that obtained for PLc, as is to be expected given the degree of amino acid identity shared by the proteins. Each of the pectate lyases has a different denaturation spectrum as followed by the change in ellipticity at 222 nm seen in Figure 9. Pectate lyase B does not display any significant secondary structural changes at concentrations of GdnHCl up to 1 M (θ_{MRW} ranging from -8682 at 0 M GdnHCl to -7398 at 1.0 M GdnHCl); however, a sharp change in observed ellipticity is seen between 1 M and 2 M GdnHCl. From 2 M GdnHCl to 6 M GdnHCl, the observed ellipticity at 222 nm ranges from -2390 to -990, indicating that a stable unfolded state has been reached. Pectate lyase C behaves quite differently, with a marked increase in observed ellipticity seen as the concentration of GdnHCl is raised from 0 M to 0.25 M ($\Delta\theta_{MRW}$ of 4499). The change in θ_{MRW} decreases as the concentration of GdnHCl is increased to 0.5 M, but a sharp increase in ellipticity is seen as the denaturant concentration is raised from 0.5 M to 1.0 M, suggesting a conformational change. As the denaturant concentration is raised from 1.0 M to 4.0 M, a steady increase in ellipticity is seen again ($\Delta\theta_{MRW}$ of 3765), suggesting yet another conformational change. The observed ellipticity does not change as the denaturant concentration is raised from 4.0 M to 6.0 M, indicative of the attainment of a completely unfolded state. The pectate lyase E protein denaturation profile is stable to concentrations of 1.0 M GdnHCl, with a slight transition ($\Delta\theta_{MRW}$ of 398) from 1.0 M to 1.5 M GdnHCl, and a sharp transition seen from 1.5 M to 2.0 M GdnHCl. A lesser decrease in ellipticity is

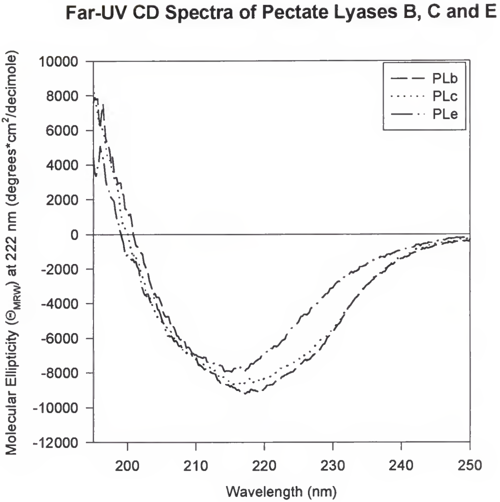


Figure 18: Far-UV circular dichroism spectra of pectate lyases B, C, and E in 10 mM sodium phosphate buffer (pH 8.0). Spectra shown are the average of 4 accumulations obtained at 25 °C on a Jasco J-500C spectropolarimeter using a 1 mm water jacketed quartz cuvette.

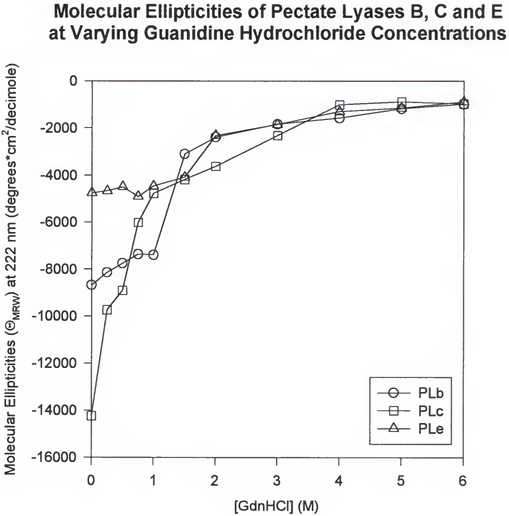


Figure 19: Molecular ellipticities as a function of GdnHCl concentration. Spectra shown are the baseline corrected average spectra from 4 accumulations.

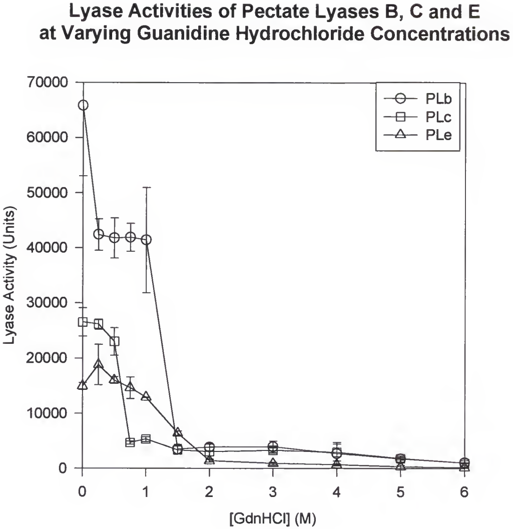


Figure 20: Pectate lyase activities at varying concentrations of guanidine hydrochloride. Activities shown are the result are the result of triplicate reactions using 10 μl of 5 μM lyase solution added to 990 μl of reaction mixture. The lyase units used to define the activities are the amount of enzyme needed to produce 1 nanomole of Δ-4,5-unsaturated bonds in 1 minute per milliliter of enzyme solution added.

seen as the denaturant concentration is raised from 2.0 M to 4.0 M ($\Delta\theta_{MRW}$ of 1021), with the observed ellipticity values stabilizing from 4.0 M to 6.0 M GdnHCl.

The changes in activities of each lyase at varying GdnHCl concentrations (Figure 20) mirror the changes seen in the circular dichroism spectra (Figure 19). Pectate lyase B shows a sharp decrease in activity as the denaturant concentration is raised to 0.25 M, followed by a retention of activity at 4.2 x 10^4 U as the GdnHCl concentration is raised from 0.25 M to 1.0 M. The activity then drops sharply from 1.0 M to 1.5 M GdnHCl, falling to 3.5 x 10 ^{3} U. Further increases in denaturant concentration to 6.0 M result in an observed activity decrease to 9.87 x 10^2 U. No changes in the activity of pectate lyase C occur as the GdnHCl concentration is raised from 0 to 0.5 M, with an observed activity 2.6 x 10^4 U detected. As the denaturant concentration is increased from 0.5 M to 0.75 M however, a sharp decrease in activity is observed, with the detectable lyase activity decreasing to 4.7 x 10 ^{3} U. As the concentration of GdnHCl increases to 6.0 M, a steady decrease in lyase activity is observed, finally reaching an activity value equivalent to that seen in the PLb samples (9.7 x 10^2 U). Pectate lyase E maintains a constant level of activity (1.4 x 10^4 U) at denaturant concentrations ranging from 0 to 1.0 M. As the concentration of GdnHCl increases further, a slow decrease in activity is observed to 2.0 M GdnHCl, where 1.4 x 10^3 U are detected. The activity decreases very slowly as the concentration of GdnHCl is increased to 6.0 M, ending at 1.5 x 10^2 detectable Units of lyase activity.

Circular dichroism measurements of pectate lyase solutions at varying temperature

The effect of temperature on the secondary structure of the pectate lyases is seen in Figure 21. Pectate lyase B and C appear to hold their native structure as the temperature is increased to 40 °C, but then a sharp decrease in observed ellipticity is seen for both

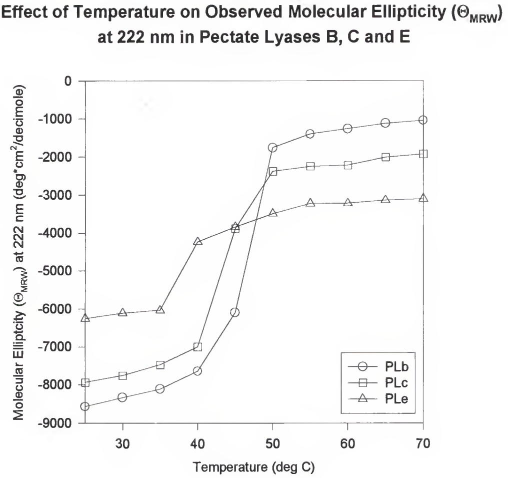


Figure 21: Observed ellipticities of pectate lyase samples at varying temperature. Spectra shown are the baseline corrected averages of 4 accumulations.

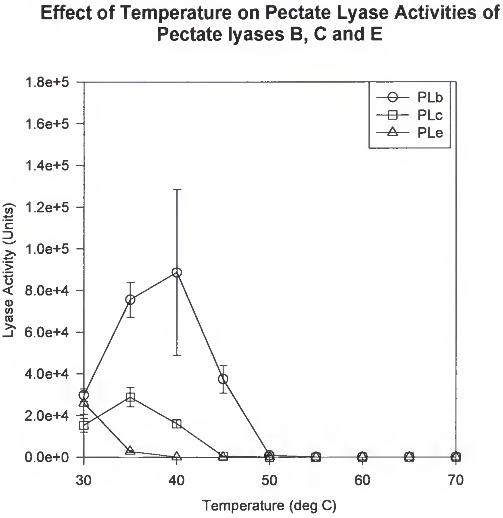


Figure 22: Pectate lyase activities at varying temperatures. Activities shown are the result of triplicate reactions using 10 μl of 0.5 μM lyase solution added to 990 μl of reaction mixture. The lyase units used to define the activities are the amount of enzyme needed to produce 1 nanomole of Δ-4,5-unsaturated bonds in 1 minute per milliliter of enzyme solution added.

enzymes. Pectate lyase C shows a steep transition, with a stable unfolded state reached by 50 °C. Pectate lyase B shows a less sharp transition to the unfolded state, but the total change in observed ellipticity is much greater. The T_m values, or the temperature at which the observed molecular ellipticity of the folded state of the protein has decreased 50% relative to the observed molecular ellipticity of the unfolded state, were calculated to be 45.6 °C for PLb and 43.9 °C for PLc. Unlike PLs b or c, pectate lyase E appears to lose secondary structure between 35 °C and 40 °C, with 30% of the ellipticity of the folded state disappearing in this temperature range. The calculated T_m value for PLe is 39.7 °C.

Enzymatic activity measurements of pectate lyase solutions at varying temperature

Pectate lyase B shows a 3-fold increase in enzymatic activity towards 0.1% PGA as the temperature is increased to 40 °C (Figure 22). The activity then drops over 50% as the temperature is increased further to 45 °C. There is only a vestige of detectable activity observed in the PLb sample as the temperature is increased further to 50 °C (782.1 U), with no activity observed at temperatures higher than 50 °C. Pectate lyase C shows a similar increase in activity as the temperature is raised, but only to 35 °C, with the activity increasing 47% as the temperature is increased 5 degrees from the initial measurement point. As the temperature is increased further to 40 °C, the observed activity decreases to that seen in the 30 °C sample, a trend that continues as the temperature is increased further to 45 °C (472 U remaining). No activity is seen in the pectate lyase C samples at temperatures higher than 45 °C. Unlike pectate lyases B or C, pectate lyase E shows no activation at slightly elevated temperatures. Instead, the observed activity decreases to 10%

of the activity at 30 °C when the temperature is increased 5 °C to 35 °C. Further increases in temperature serve to remove any observable activities in the PLe sample.

Discussion

Circular dichroism measurements of guanidine hydrochloride denatured pectate lyase solutions

The folding motif possessed by the pectate lyase family of enzymes would seem to indicate a large amount of hydrogen bonding, and therefore should require a high concentration of a chaotropic agent such as GdnHCl to significantly disrupt the overall structure of the enzymes. This is not observed to be the case however, with PLs b, c and e showing significant disruption (50% of the native structure removed relative to the observed ellipticity for the unfolded state) of secondary structure at 1.25 M GdnHCl, 0.75 M and 1.75 M, respectively. The observation that PLe retains more of its structure is also surprising, given the observation that it possesses fewer α-helices and β-sheets as a percentage of the total secondary structural elements in the protein (Sieber et al., 1995). The difference between PLb and PLc with respect to the denaturant concentration needed to remove 50% of the observed secondary structure is also surprising, given the degree of amino acid identity shared by the two enzymes. This difference is not an experimental artifact however, as the activity data obtained for PLb and PLc correlates nicely with the observed ellipticity data. The drop in lyase activity seen for PLc between 0.5 M and 0.75 M GdnHCl is mirrored in the observed ellipticity in the same concentration range. The same is true of pectate lyase B, with the stable, folded state seen in the circular dichroism spectra up to 1.0 M GdnHCl also seen in the retention of the same level of activity through the same concentration range. This begs the obvious however; the structure of the enzymes and their respective enzymatic activities are directly related. PLb and PLc appear to be able to accept a small degree of structural disruption (PLb tolerating more), until a critical concentration of denaturant is reached, at which the activity drops as rapidly as the tertiary structure of the enzyme deteriorates. The same does not appear to be true in the case of pectate lyase E. Although the molecular ellipticity of the enzyme appears to be stable at GdnHCl concentrations up to 1.5 M, the activity steadily decreases across the same concentration range. In other words, the enzyme loses its activity at much lower chemical denaturant concentrations, suggesting that the chemical moieties involved in the catalytic process have very little tolerance for spatial perturbation. This is not unexpected for an enzyme that is highly specific with respect to the large, polyanionic substrate it recognizes and has evolved a processive manner to depolymerize the substrate to which it binds. It may be that at non-denaturing concentrations of GdnHCl, either the side chains of the amino acids involved in binding substrate or in the catalytic events of the chemical reaction catalyzed by the enzyme are shifted slightly, resulting in a non-functional state.

Circular dichroism measurements of pectate lyase solutions at varying temperature

If GdnHCl disrupts protein structure by removing the hydrogen bond network existing throughout the protein, then temperature may well be considered a global denaturant, by increasing the energy of the entire system, protein and solution. The thermal denaturation studies also serve as a better measuring stick with respect to the environmental function of the enzymes, as the host organism for *Erwinia* species lives in an arena that is subject to thermal extremes. Enzymes that function in this environment are expected to have some degree of thermal tolerance, and that is seen in pectate lyases B and C. The thermal denaturation profiles obtained for each enzyme (Figure 21) are similar, as is to be expected given their sequence identity, with PLb showing more thermal tolerance than PLc (1.7 °) until 50% of the enzyme pool is denatured. Pectate lyase E shows the least thermal tolerance, with 50% of the enzyme denatured at 39.7 °C. It is interesting to note that; unlike the GdnHCl experiments where the ellipticities of the denatured proteins converged, in the thermal studies none of the ellipticities converged, suggesting that a different structural state is reached when heat is the denaturing agent.

Enzymatic activity measurements of pectate lyase solutions at varying temperature

A comparison of the lyase activity data in response to increasing temperature and the decrease in molecular ellipticity observed under the same conditions shows that in pectate lyases B and C, some change in structure caused by heat is not detrimental to the activities of the enzyme, but rather the opposite. Pectate lyase C shows maximal activity at 35 °C, well below the transition temperature seen in the circular dichroism studies. The activity returns to that seen in samples incubated at 30 °C when the temperature is raised still more to 40°C, which is just before a thermally induced structural change occurs in the enzyme. This suggests that some slight structural changes can be tolerated by the enzyme, but not many, as the temperature optimum for the depolymerization reaction begins to drop as the transition temperature is reached. This is not the case with PLb, which shows an increase in activity up to the transition region (40°C to 50 °C). An increase in the temperature should cause increases in rates of substrate and product diffusion, but also a more rapid "breathing" or increased flexibility of the protein molecules. It would seem that increasing the solution dynamics of the pectate lyase B molecule does not detrimentally affect the enzyme, but rather increases the number of reactions that it can catalyze per unit time. The same is not true for pectate lyase E, which shows a constantly decreasing enzymatic activity as the temperature is raised above 30 °C. As was seen in the guanidine hydrochloride denaturation experiments, slight perturbations in the fine structure of PLe result in detectable decreases in enzymatic activity, something that would be expected for a highly specialized enzyme.

CHAPTER 4

CONFORMATIONAL DIFFERENCES BETWEEN PECTATE LYASES B, C AND E AS DETERMINED BY LIMITED PROTEOLYSIS

Introduction

Proteases are enzymes catalyzing the hydrolysis of peptide bonds within a protein. Some proteases possess a high degree of specificity with respect to the bonds that can be recognized and subsequently cleaved, and as such can be invaluable tools in the elucidation of the tertiary structures of proteins (Price and Johnson, 1989). Given the similarities in the folding motif shared by the gene products of the *pel*BC gene cluster and the *pel*ADE gene cluster, the observed functional differences (Preston et al., 1993) must arise from evolutionary fine tuning of discrete structural elements comprising the β-helical fold. The resistance of virulence factors such as the pectate lyases to protease induced inactivation could be a key factor influencing the success of a pathogenic organism such as *Erwinia chrysanthemi* EC16 (Dalling, 1986). Previous experiments have determined differences in relative susceptibilities to proteolysis between pectate lyases B and C (Hurlbert, 1996), but have not rigorously examined the same properties in pectate lyase E. The evolved differences in sequence (16% for PLb/PLc and over 60% for PLb-c/PLe) may translate not only into the functional differences previously alluded to, but a differential susceptibility to proteolytic attack that would allow the infectious process to continue, thereby ensuring both the continued survival of the bacterial species and the retention of several homologous genes on the bacterial chromosome. With these factors in mind, the following experiments were designed to determine the differences in protease susceptibility of pectate lyase B, C and E, as well as the differences in solution structure that give rise to the observed protease sensitivities.

Materials and Methods

Proteolytic reactions

Solutions of each of the pectate lyases purified as described previously (Chapter 2) were subjected to limited proteolysis by incubation with the site specific endoproteinases AspN, GluC and Trypsin. A stock solution of AspN (Sigma Cat # P-3303) in 50 mM sodium phosphate buffer pH 7.0 was made such that the final concentration of endoproteinase was 1 μg/μl (Bentz, et al., 1990). Aliquots of this solution were added to solutions of PLb, c or e in 50 mM sodium phosphate buffer pH 7.0 such that the final concentration ratio of pectate lyase to endoproteinase was 100 : 1. For reactions employing GluC (Sigma Cat # P-6181) (Tomasselli et al., 1986) or trypsin (Sigma Cat # T-8658) (Eggerer, 1984), stock solutions of each endoproteinase were prepared in 100 mM ammonium bicarbonate buffer such that the final concentration of each protein was 1.0 μg/μl. Aliquots of the proteases were added to solutions of each pectate lyase such that the final concentration ratio of PL to endoproteinase was 100 : 1.

Capillary electrophoretic analysis of proteolytic reactions

Each of the proteolytic reactions were analyzed on a Beckman P/ACE system 5510 capillary electrophoresis unit equipped with a 75 μm I.D. x 57 cm eCAP fused silica capillary. The capillary was equilibrated in 50 mM phosphoric acid pH 2.5 prior to a 5 second pressure injection from the proteolytic reaction vial. Separation was performed in 50 mM phosphoric acid, pH 2.5, at 20 kV with a 0.17 sec ramp time for 25 minutes accompanied by inline monitoring of the absorbance at 214 nm for peptide and/or protein detection. Samples were run immediately upon addition of the protease to the reaction mixture (0 time), and every 30 minutes thereafter to 3 hours of reaction, with samples then analyzed every hour from 4 hours to 6 hours of digestion. The capillary was rinsed with 0.1 N NaOH and GVWP filtered deionized water between each injection/separation. The entire system was controlled and data were analyzed using the Beckman System Gold for Windows 95 (ver 1.0) software generously supplied by Beckman, Inc. Selected electropherograms for each pectate lyase and endoproteinase are found in Figures 23-31.

MALDI-TOF mass spectrometry of proteolytic samples

After the aliquots were removed from the proteolytic reaction mixtures for lyase activity analysis, another 0.5 μl aliquot was removed and placed in 4.5 μl of 0.2% TFA, with the resulting solution mixed by aspiration. These samples were frozen until analyzed on a Perseptive Biosystems Voyager MALDI-TOF mass spectrometer. Sample preparation was accomplished by unthawing the samples, removing 2.0 μl from the microcentrifuge tube, mixing it with 2.0 μl of a saturated solution of matrix (α-cyano-4-hydroxycinnamic acid (Beavis et al., 1992) in 40% acetonitrile / 10% formic acid), and placing 2.0 μl of the resulting solution on a 100 spot, stainless steel sample plate. Upon completion of sample spotting, the plate was allowed to air dry at room temperature, at which point it was loaded into the mass spectrometer. The instrument was operated under the following parameters: Linear mode, 30 kV accelerating voltage, 90% grid voltage, 0.2% guide wire voltage, low mass gate on). Spectra obtained were analyzed with the GRAMS/386 software package (Galactic Industries, Inc.) (Figures 32-41).

HPLC separations of peptides produced in selected proteolytic reaction mixtures

Based upon the susceptibilities of each lyase to proteolytic attack as determined by the appearance of peptide peaks in the capillary electropherograms, 6 hour digests of each PL by AspN, as well as a 3 hour digest of PLc by GluC, were selected for peptide purification by reverse-phase HPLC. The peptide separations were performed by injecting 150 μl of a 15-fold dilute sample of each proteolytic reaction mixture into a Hewlett-Packard 1090 liquid chromatograph equipped with a C_4 column (0.21 x 25 cm, Vydac, Inc.). The sample was eluted from the column with a solvent gradient (0.15 ml/min flowrate, Solvent A = 0.1% TFA in dH_2O, Solvent B = 0.07% TFA in 80% acetonitrile, linear gradient lasting 100 minutes from 5% to 70% Solvent B) and the eluted peptides were detected by an inline UV detector monitoring the change in absorbance at 220 nm. Peak fractions were collected manually into microcentrifuge tubes and analyzed by MALDI-TOF mass spectrometry under the previously described conditions. Chromatograms are presented in Figures 42, 43, 44 and 45.

Identification of proteolytic cut sites within each pectate lyase molecule

To identify the most susceptible regions to proteolytic attack in each pectate lyase molecule, two approaches were employed. In the first method, the capillary electrophoresis data was used to quantify the formation of stable peptide products created by proteolytic cleavage of each pectate lyase, by identifying peptide peaks in the electropherograms that were seen at early times and found to persist during the time course of the study. The kinetic MALDI-TOF MS data served to identify the masses of those peptides, data that was then used to identify the sequences of the peptides using the Protein Analysis Workbench program (PAWS, freeware version 8.3.0.6, ProteoMetrics, Inc, 1998). The program uses the known specificity of select proteases to search through the input protein sequence for target peptide masses (Table 4). The cut sites identified in this manner were then mapped onto the three-dimensional structure of each protein using WebLab Viewer Pro (ver 3.10, Molecular Simulations Inc, 1998). In the second method, amino terminal amino acid sequencing was used to identify the sequence of peptide products purified from the proteolytic digests. Candidates for peptide sequencing were identified based upon their peak areas and molecular weights as determined by MALDI-TOF MS. Peak fractions found to contain peptides identified in the kinetic MALDI-TOF MS experiments as being generated in an initial cleavage event were submitted to the ICBR Protein Core for amino terminal sequencing. Cut sites within each lyase are given in Table 4.

Results

Capillary electrophoretic analysis of proteolytic reactions

As can be seen in Figures 26-31 , capillary electrophoretic analysis of the proteolytic reactions is an effective tool for gauging the extent of degradation of each pectate lyase. In reactions involving AspN and PLb (Figure 23), three peaks are seen after an hour of incubation. These peaks are seen throughout the time course of the study, with intact PLb molecules degraded to peptides comprising the peaks seen at migration times of 9.9 and 11.6 minutes. Upon the conclusion of the 6 hour study of PLb digestion by AspN, 36% of the intact enzyme (based upon peak area) was cleaved into peptide fragments. In Figure 24, two peaks are seen in the electropherogram obtained at zero time. The peak at 11.3 minutes has been confirmed to be an artifact generated under the experimental conditions used. The sample of PLc used in the experiments showed no contaminants when analyzed by reverse phase HPLC, MALDI-TOF MS or SDS-PAGE, and was shown

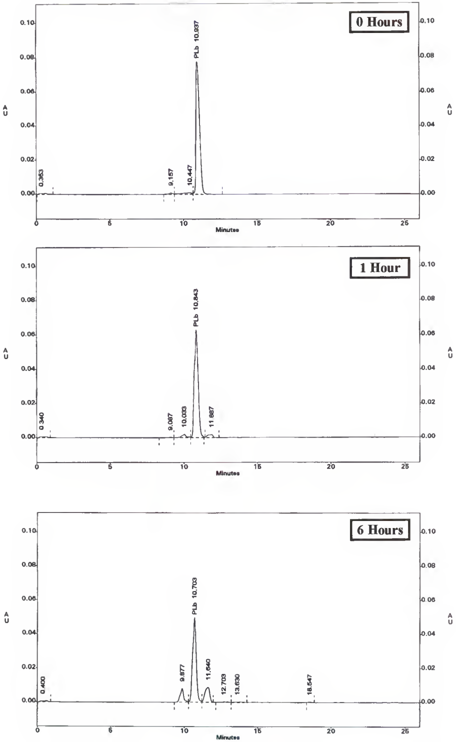


Figure 23: Capillary electropherograms of a PLb/AspN digest sampled at 0, 1 and 6 hours after the addition of protease.

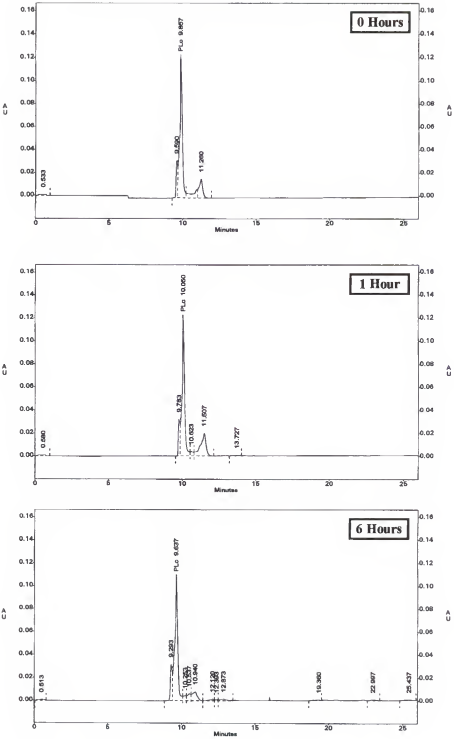


Figure 24: Capillary electropherograms of a PLc/AspN digest sampled at 0, 1 and 6 hours after addition of protease.

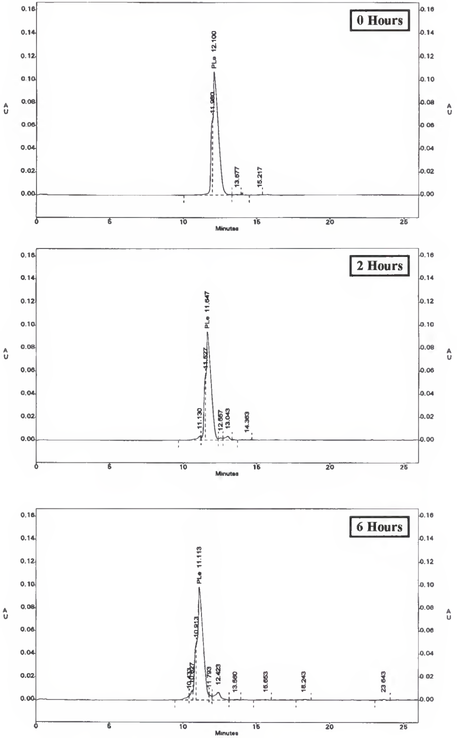


Figure 25: Capillary electropherograms of a PLe/AspN digest sampled at 0, 2 and 6 hours after addition of protease.

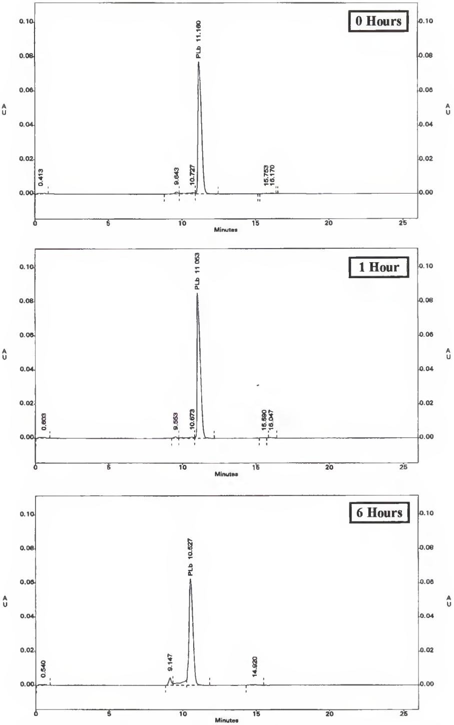


Figure 26: Capillary electropherograms of a PLb/GluC sampled at 0, 1 and 6 hours after the addition of protease.

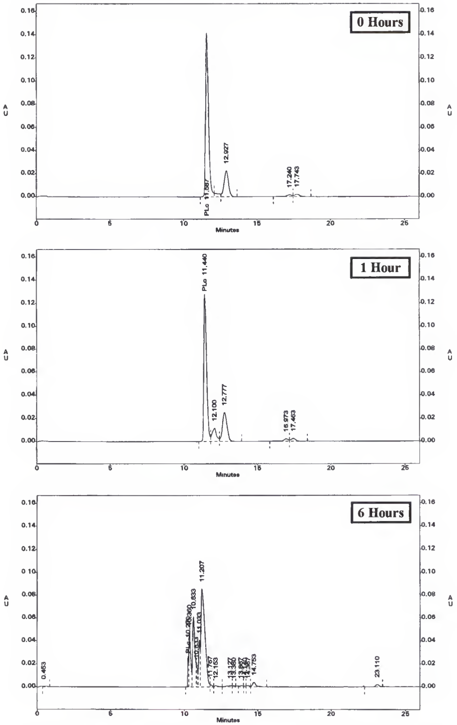


Figure 27: Capillary electropherograms of a PLc/GluC digest sampled at 0, 1 and 6 hours after addition of protease.

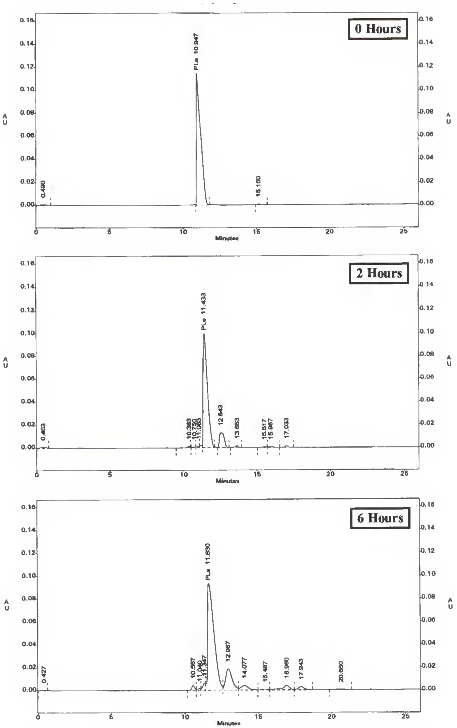


Figure 28: Capillary electropherograms of a PLe/GluC digest sampled at 0, 2 and 6 hours after addition of protease.

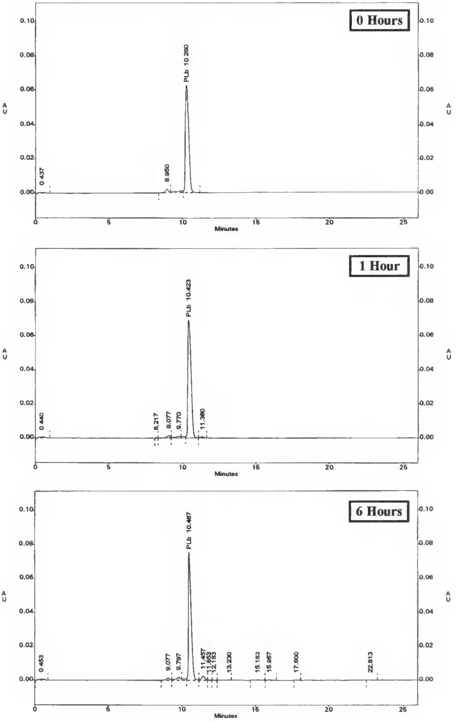


Figure 29: Capillary electropherograms of a PLb/trypsin sampled at 0, 1 and 6 hours after the addition of protease.

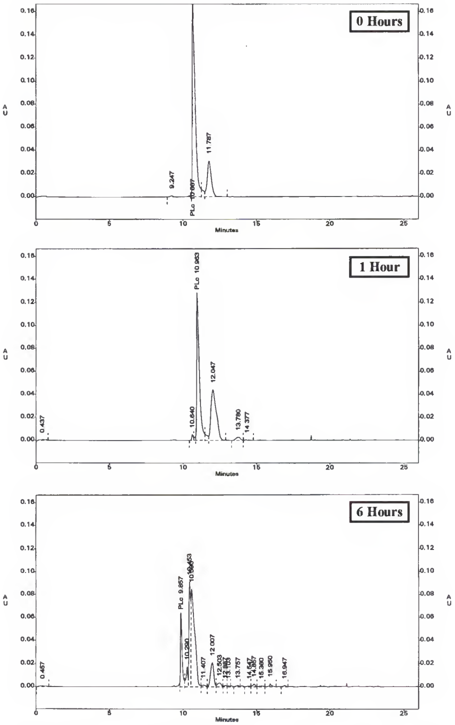


Figure 30: Capillary electropherograms of a PLc/trypsin digest sampled at 0, 1 and 6 hours after addition of protease.

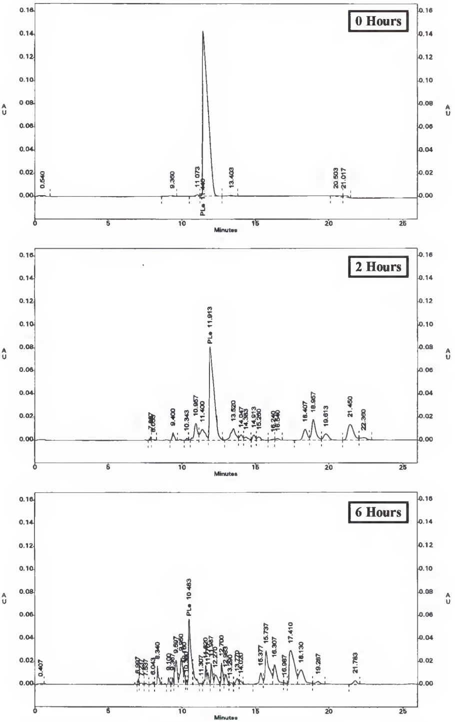


Figure 31: Capillary electropherograms of a PLe/trypsin digest sampled at 0, 2 and 6 hours after addition of protease.

to be properly folded by CD spectroscopy. An hour after the addition of the endoprotease, peaks are seen to appear as shoulders to the intact PLc peak at 10.1 minutes and 11.5 minutes. After 6 hours of digestion, the peak area of the intact PLc peak has decreased 7%, with the non-resolved shoulder still seen, and the peak at 11.3 minutes has split into two peaks seen at migration times of 10.9 minutes and 10.5 minutes. Pectate lyase E is also found to be susceptible to AspN cleavage as seen in Figure 25. An hour after the addition of protease, a non-resolved shoulder is seen attached to the intact PLe peak at 11.9 minutes, in addition to the peaks seen at 11.4 minutes, 12.9 minutes and 13.4 minutes. After 6 hours of digestion, many of the previously resolved peaks have merged, with 3 non-resolved peaks seen as part of the intact PLe peak at 11.1 minutes, and the peaks seen at 12.9 and 13.4 minutes in the 1 hour sample merging to form a single resolved peak at 12.4 minutes. After 6 hours of digestion, 10% of the native PLe molecules in the reaction have been proteolytically cleaved to peptide products.

Pectate lyase B is seen to be resistant to proteolytic cleavage by the endoprotease GluC (Figure 26). Other than the peak assigned to native PLb, the only other detected peak in the reaction mixture after 6 hours of digestion has a migration time of 9.15 minutes, but this peak is also seen in the zero time sample and could belong to the protease. The area of this peak does increase slightly during the time course of the study and the native PLb peak is seen to broaden in samples from later times, a phenomenon that could be due to electrolytic breakdown of the running buffer. Pectate lyase C does not appear to share the resistance of its homologue to proteolytic attack by GluC (Figure 27). After an hour of incubation with GluC, 18% of the intact PLc molecules have been degraded to peptides detected at 12.1 and 12.8 minutes. After 6 hours of digestion by GluC, 73% of the intact PLc (10.4 minute migration time) has been degraded, with prominent peaks detected at 10.6

and 11.2 minutes. Pectate lyase E is also highly susceptible to proteolytic degradation by GluC as confirmed by the 36% decrease in peak area seen for the intact enzyme (11.2 minutes) after 2 hours of incubation (Figure 28). A double peak is seen at 12.6 minutes in the electropherogram from the 2 hour sampling time. After 6 hours of digestion, the intact PLe peak has disappeared and been replaced with a double peak at 11.4 minutes with two, smaller unresolved peaks seen just before the doublet. The previously seen split peak at 12.6 minutes has fused to form a single peak with a 12.7 minute migration time. Smaller peaks are also seen at 10.4, 13.8 and 17.2 minutes.

Trypsin appears to degrade PLb, with a 6% decrease in the peak area for intact PLb seen after an hour of digestion by the endoprotease, and an 18% decrease in peak area seen after 6 hours of digestion (Figure 29). Small peaks are seen in all electropherograms at 9.1, 9.8, and 11.4 minute migration times. PLc is much more sensitive to proteolytic cleavage by trypsin as seen in Figure 30. After an hour of digestion, the peak area of intact PLc (10.7 minutes at 0 time, 10.9 minutes at the 1 hour sampling time) is seen to decrease by 30%, with a large increase shown in the peak at 12.0 minutes (11.8 minutes in the 0 time sample). After 6 hours of digestion by trypsin, a 77% decrease in peak area of intact PLc is seen. The peak detected at a migration time of 11.8 minutes in the 1 hour electropherogram is comprised of at least 3 peaks in the 6 hour sample, with an average migration time of 10.4 minutes. A large peak is seen at 12 minutes along with many smaller peaks seen with migration times of 12.5 to 23 minutes. As seen in Figure 31, pectate lyase E shows the greatest susceptibility to trypsin under the conditions used. The intact enzyme peak with a migration time of 11.7 minutes in the 2 hour electropherogram is 10% smaller than the intact PLe control peak, and after 6 hours of digestion, is 72% smaller than the same peak in the control sample. In the 2 hour electropherogram, peaks are seen with migration times of

9.4, 13.4, 18.1, 18.7, 19.5 and 21.0 minutes. At the 6 hour sampling time, many large peaks are seen at various migration times, with the two most prominent peaks seen at 15.7 and 17.4 minutes (18.7 and 21.0 minutes in the 2 hour electropherogram, respectively).

MALDI-TOF mass spectrometry of proteolytic samples

The mass spectrum of the pectate lyase B/AspN reaction sampled at time zero in Figure 32 shows the intact enzyme with a calculated mass of 38018 Da, as well as the multiply charged species ($M/2H^+$, $M/3H^+$ and $M/4H^+$) at 19042, 12754 and 9632 Da. After 30 minutes of reaction, additional peptides with masses of 16816 Da and 21298 Da are detected in the reaction mixture. These peptides are also seen in the 6 hour sample, with increased intensity, as well as peptides having masses of 10710 Da, 8352 Da, 7153 Da, 5561 Da and 4950 Da. Figure 33 shows the zero time, 30 minute and 6 hour mass spectra from the pectate lyase C/AspN reaction. At time zero, the intact PLc mass of 37849 Da is observed, as well as the multiply charged species with masses of 18929 Da and 12759 Da ($M/2H^+$ and $M/3H^+$, respectively). In the 30 minute mass spectrum for the same reaction, these same masses are seen as well as newly observed masses of 7605 Da, 8451 Da and 16816 Da. In the 6 hour sample, the 16903 Da mass is still seen, joined by detected mass fragments of 20201 Da, 8533 Da (which could be the $M/2H^+$ species of the 16816 Da peptide), 10225 Da, 4984 Da, 3717 Da and 3209 Da. Pectate lyase E/AspN mass spectral data at zero time, 30 minutes and 6 hours of incubation are given in Figure 34. At time zero, as was the case with the other lyase samples, the mass of intact PLe is seen (38188 Da) along with the signals from the multiply charged species of the intact enzyme ($M/2H^+$ at 19185 Da and $M/3H^+$ at 12846 Da). In the 30 minute sample, these same species are seen, with 2 new masses observed at 25514 Da and 13403 Da. In the 6 hour sample, the intact

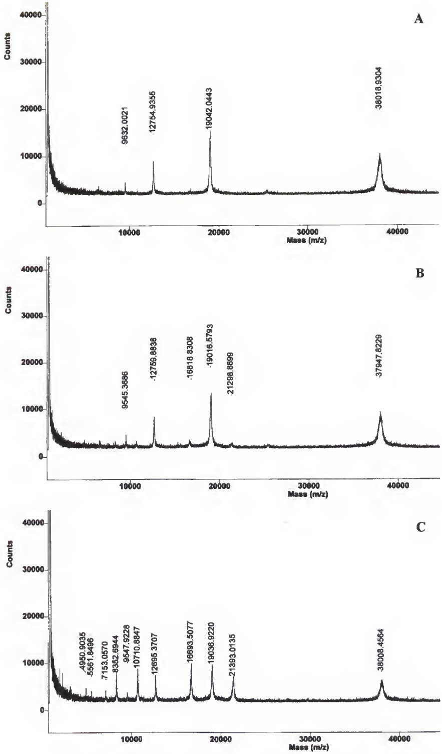


Figure 32: MALDI-TOF mass spectrograms of 0 (A), 0.5 (B) and 6 (C) hour digests of pectate lyase B using the endoproteinase AspN. α-Cyano,4-deoxy cinnamic acid was used as a matrix, mixed 1:1 with sample and placed on a 100 position sample plate for analysis on a Perseptive Biosystems Voyager Matrix Assisted Laser Desorption Time of Flight Mass Spectrometer. Data were analyzed with the GRAMS/386 software package (Galactic Industries).

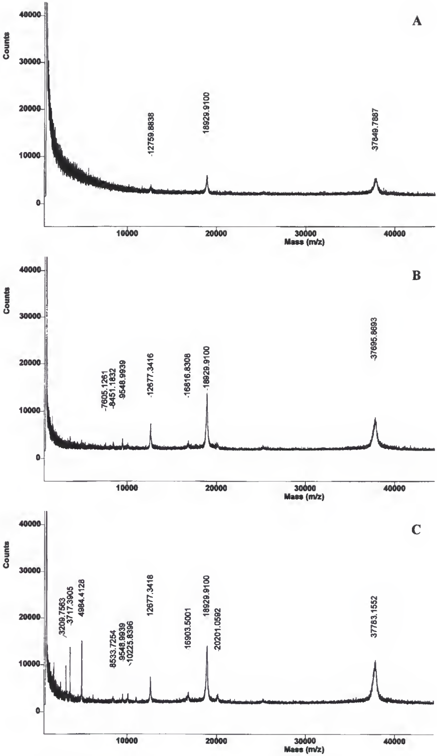


Figure 33: MALDI-TOF mass spectrograms of 0 (A), 0.5 (B) and 6 (C) hour digests of pectate lyase C by the endoproteinase AspN. Samples were prepared and data were collected and analyzed by the methods described in Figure 32.

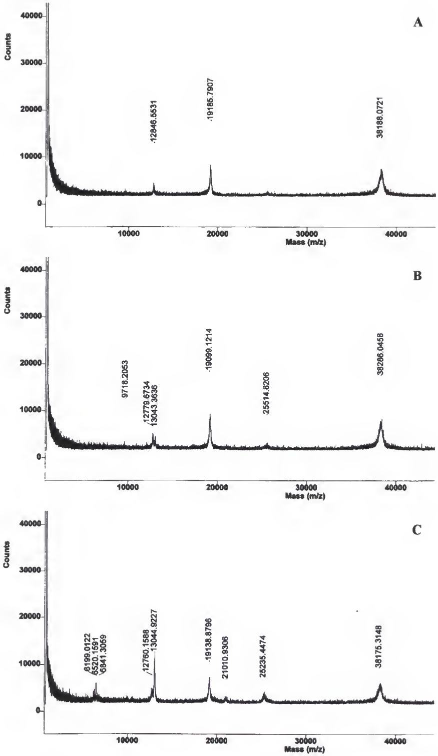


Figure 34: MALDI-TOF mass spectrograms of 0 (A), 0.5 (B) and 6 (C) hour digests of pectate lyase E by the endoproteinase AspN. Samples were prepared and data were collected and analyzed by the methods described in Figure 32.

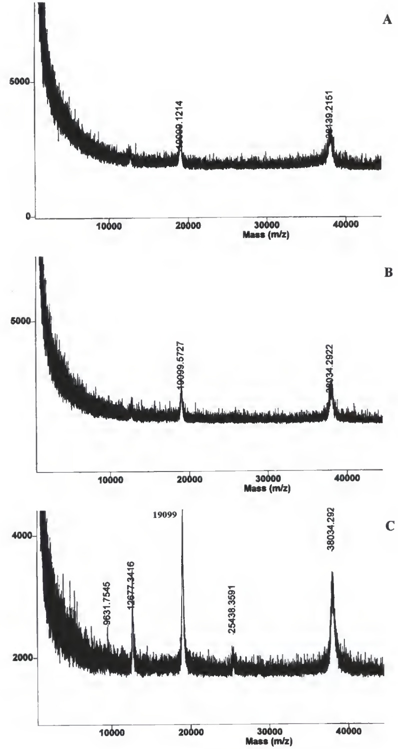


Figure 35: MALDI-TOF mass spectrograms of 0 (A), 0.5 (B) and 6 (C) hour digests of pectate lyase B using the endoproteinase GluC. α-Cyano,4-deoxy cinnamic acid was used as a matrix, mixed 1:1 with sample and placed on a 100 position sample plate for analysis on a Perseptive Biosystems Voyager Matrix Assisted Laser Desorption Time of Flight Mass Spectrometer. Data were analyzed with the GRAMS/386 software package (Galactic Industries).

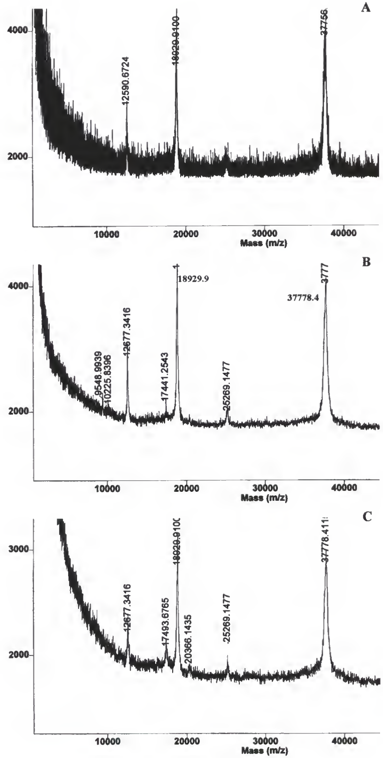


Figure 36: MALDI-TOF mass spectrograms of 0 (A), 2 (B) and 6 (C) hour digests of pectate lyase C by the endoproteinase GluC. Samples were prepared and data were collected and analyzed by the methods described in Figure 5A.

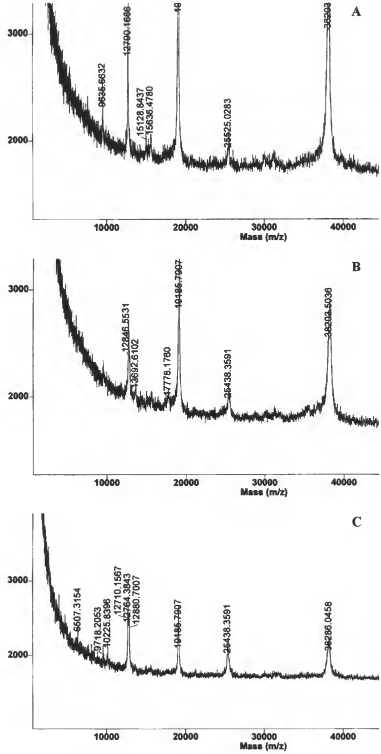


Figure 37: MALDI-TOF mass spectrograms of 0 (A), 1 (B) and 6 (C) hour digests of pectate lyase E by the endoproteinase GluC. Samples were prepared and data were collected and analyzed by the methods described in Figure 35.

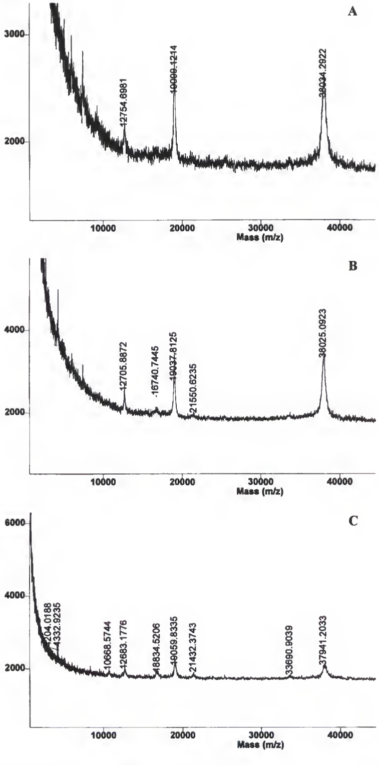


Figure 38: MALDI-TOF mass spectrograms of 0 (A), 0.5 (B) and 6 (C) hour digests of pectate lyase B using the endoproteinase trypsin. α-Cyano,4-deoxy cinnamic acid was used as a matrix, mixed 1:1 with sample and placed on a 100 position sample plate for analysis on a Perseptive Biosystems Voyager Matrix Assisted Laser Desorption Time of Flight Mass Spectrometer. Data were analyzed with the GRAMS/386 software package (Galactic Industries).

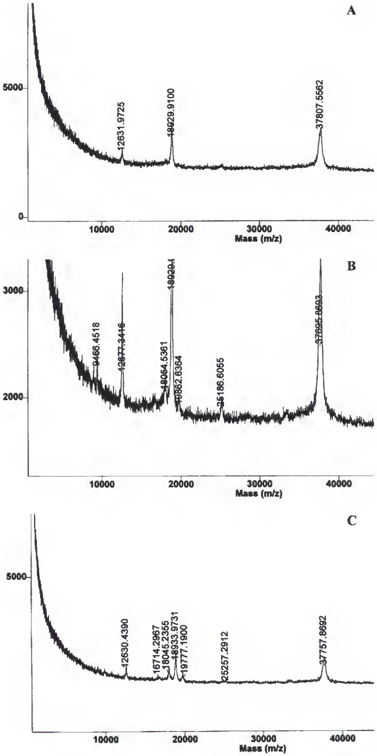


Figure 39: MALDI-TOF mass spectrograms of 0 (A), 3 (B) and 6 (C) hour digests of pectate lyase C by the endoproteinase trypsin. Samples were prepared and data were collected and analyzed by the methods described in Figure 38.

A

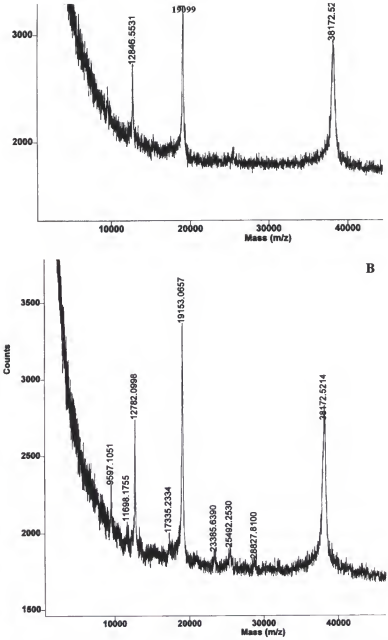


Figure 40: MALDI-TOF mass spectrograms of 0 (A) and 0.5 (B) hour digests of pectate lyase E by the endoproteinase trypsin. Samples were prepared and data were collected and analyzed by the methods described in Figure 38.

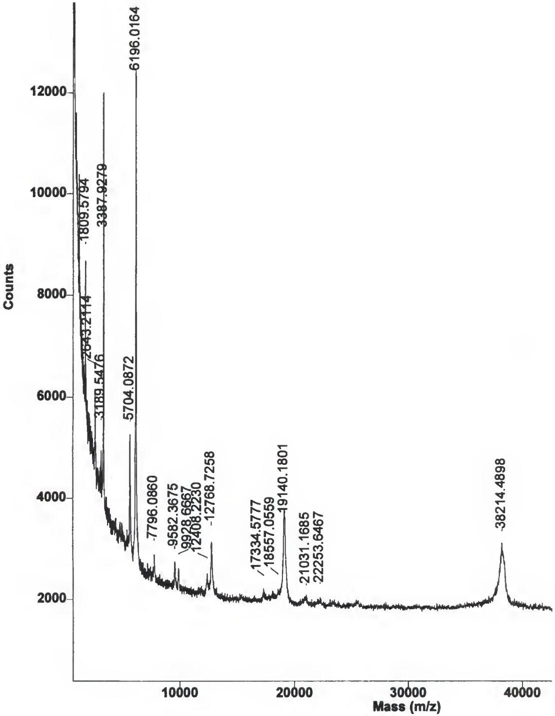


Figure 41: MALDI-TOF mass spectrogram of a 6 hour digest of pectate lyase E by the endoproteinase trypsin. The sample was prepared and data were collected and analyzed by the methods described in Figure 38.

protein and the multiply charged derivative species can still be seen, as can peptides with masses of 25235 Da, 21010 Da, 13044 Da (at much higher intensity than seen in the 30 minute sample), 6841 Da, 6520 Da and 6199 Da.

The mass spectra obtained from the GluC digestions of each of the pectate lyases can be found in Figures 35-37. As was seen in the capillary electropherograms of the PLb/GluC reaction (Figure 26), no peptide products are seen in the samples at any of the times studied (Figure 35). The only masses seen in the spectra from time zero to 6 hours are those of intact PLb (38034 Da) and the multiply charged species ($M/2H^+$ at 19099 Da and $M/3H^+$ at 12672 Da), confirming that pectate lyase B is not cleaved by GluC under the conditions studied. No peptides are seen in the pectate lyase C/GluC reaction until 2 hours of digestion, when peptides of mass 25269 Da, 17441 Da, 10225 Da are seen in addition to the intact enzyme mass signal (37778 Da) and its multiply charged species ($M/2H^+$ at 18929 Da, $M/3H^+$ at 12677 Da and $M/4H^+$ at 9548 Da). In the 6 hour sample, peptide masses of 17493 Da, 20366 Da and 25269 Da are seen (Figure 36). Pectate lyase E/GluC mass spectra are found in Figure 37. At time zero, a mass signal of 38203 Da is seen for the intact protein, with the multiply charged species seen at 19185 Da ($M/2H^+$), 12790 Da ($M/3H^+$) and 9635 Da ($M/4H^+$). In addition to these peaks, masses of 25525 Da, 15636 Da and 15128 Da are also seen. In the 1 hour sample, the intact protein and multiply charged species are observed, as well as the peptide masses of 25438 Da and 17778 Da and a peptide mass of 13692 Da. In the mass spectrum of the 6 hour sample, the intact protein and its ionized derivative masses can still be seen, as can the peptide masses seen in the hour sample of 25444 Da, 17787 Da and 13694 Da. Peptide masses of 12904 Da, 11776 Da and 10179 Da are also seen in the mass spectrum from this sample time.

The sample removed from the pectate lyase B/trypsin reaction mixture at time zero shows the mass signals from the intact enzyme (38034 Da) as well as the multiply charged species of the enzyme (19099 Da and 12754 Da) as seen in Figure 38. After 30 minutes of incubation, two other peptides are seen in the sample, having masses of 16740 Da and 21550 Da, respectively. In the sample removed from the reaction vial after 6 hours, four additional peptides with masses of 33690 Da, 10668 Da, 4332 Da and 4204 Da are also observed in the mass spectrum. In the PLc/trypsin sample taken at zero time, the intact mass signal and the charged derivative mass signals can be seen (Figure 39) as well as a mass signal at 25269 Da. These signals remain the only detected masses until the 3 hour sample, when peptides of mass 18054 Da and 19862 Da are observed in the mass spectrum. After 6 hours of digestion, peptide masses of 16714 Da, 18045 Da and 19777 Da are detected in the reaction mixture, as well as the mass signals derived from the intact enzyme. Pectate lyase E was the most susceptible pectate lyase to trypsin cleavage as determined by capillary electrophoresis, and this is reflected in the mass spectra of samples taken from the reaction seen in Figures 40 and 41. At zero time, only the signals from the intact enzyme are detectable. However, by 30 minutes of digestion, peptide masses of 11698 Da, 17335 Da, 23385 Da, 25492 Da and 28827 Da are seen in the reaction mixture. After 6 hours of digestion by trypsin (Figure 41), pectate lyase E has been cleaved to a wide range of peptide sizes, with masses of 1809 Da, 2643 Da, 3189 Da, 3387 Da, 5704 Da, 6196 Da, 7796 Da, 9928 Da, 12408 Da, 17334 Da, 18557 Da, 21031Da and 22253 Da detected in the sample.

HPLC Separations of peptides produced in selected proteolytic reaction mixtures

As seen in Figure 42, several peptides were produced during the digestion of PLb with AspN. Based upon the area of each peak, peaks 1, 4, 7, 12, 13, 15, 16 and 17 were selected for MALDI-TOF MS analysis with the observed masses reported over each peak. The peptide masses seen in the 30 minute sample (16816 Da and 21298 Da) are found in peaks 17 and 13 respectively. Based upon this observation, peak 13 was selected for amino-terminal sequencing to identify the amino acid sequence of the 21298 Da peptide. The chromatogram from the HPLC separation of the 6 hour PLc/AspN digestion is found in Figure 43. The two major peaks visible were analyzed by MALDI-TOF MS with peak 1 found to contain two peptides, one with a mass of 6739 Da and the other with a mass of 20130 Da. Intact enzyme found in the second peak. The fraction containing peak 1 was selected for amino terminal sequencing to accurately determine the amino acid sequence of the 20130 Da peptide. In chromatogram obtained from the HPLC separation of the 6 hour digest of PLe by AspN (Figure 44), 4 large peaks are seen. Peak 1 was found to contain the 13057 Da peptide seen after 1 hour of digestion in the kinetic MALDI-TOF MS experiments. Peak 2 contained two peptides with observed masses of 19775 and 21042 Da. The only detectable mass signal in peak 4 was that of intact enzyme. The peak at 87 minutes in the chromatogram is an unknown component seen at the end of every chromatographic run, even when a blank solution of water is run through the column. From the peak fractions obtained, peak 1 was selected as a candidate for amino terminal sequencing. The chromatogram from the separation of the 3 hour digestion of PLc by GluC is found in Figure 45. Peaks 1, 3 and 4 were analyzed by MALDI-TOF MS. Peak 1 contained two peptides with observed masses of 6845 Da and 20415 Da. The 20415 Da peak was seen in the 2 (although it was not integrated) and 6 hour kinetic MALDI-TOF MS samples of the same digest. Peak 4 consists of a small amount of intact enzyme, a 10198 Da peptide and a 17465 Da peptide also seen in the mass spectrum of the same sample after 30 minutes of digestion. Peak 5 contains a strong mass signal for intact enzyme, a much

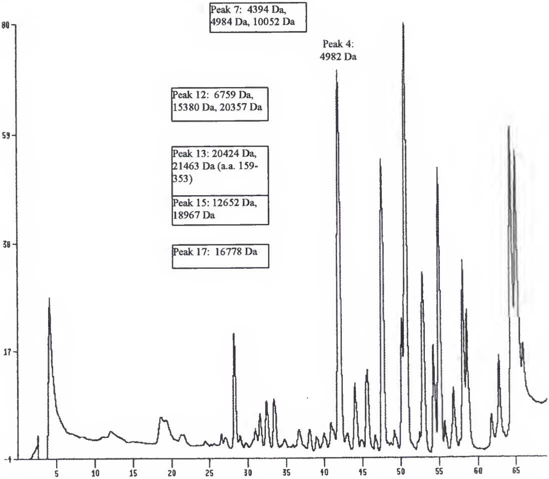


Figure 42: Reverse phase HPLC separation of a 6 hour AspN digest of pectate lyase B. Sample was prepared by adding 10 μl of a the PLb/AspN proteolytic reaction solution described in Materials and Methods to 140 μl of deionized water which was then injected onto a C_4 column (Vydac, Inc., 0.21 x 25 cm) and eluted with a 70 minute linear gradient of 0.1% TFA in 4% acetonitrile to 0.08% TFA in 56% acetonitrile. Peaks were detected using an inline UV detector set to 220 nm. The amino acid sequence of the 21 kDa peptide in peak 13 is given as determined from amino acid sequencing.

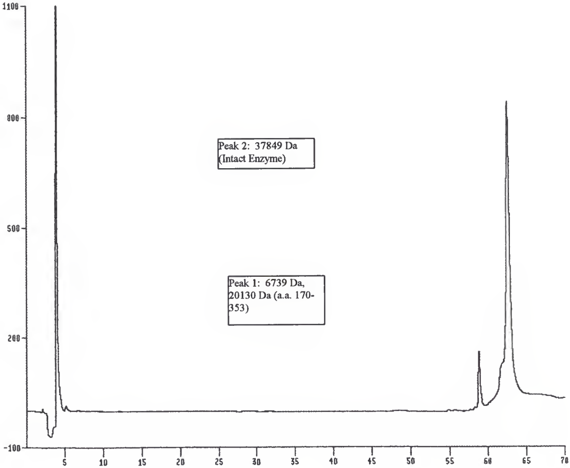


Figure 43: Reverse phase HPLC separation of a 6 hour AspN digest of pectate lyase C. Sample was prepared by adding 10 μl of a the PLc/AspN proteolytic reaction solution described in Materials and Methods to 140 μl of deionized water which was then injected onto a C_4 column (Vydac, Inc., 0.21 x 25 cm) and eluted with a 70 minute linear gradient of 0.1% TFA in 4% acetonitrile to 0.08% TFA in 56% acetonitrile. Peaks were detected using an inline UV detector set to 220 nm. The amino acid sequence of the 20 kDa peptide in peak 1 is given as deduced from the molecular weight and verified by amino acid sequencing.

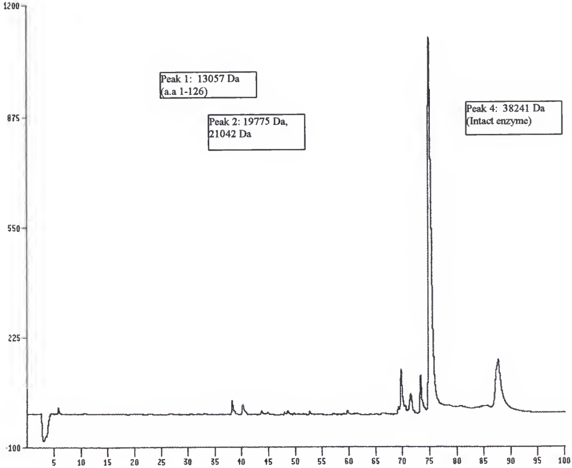


Figure 44: Reverse phase HPLC separation of a 6 hour AspN digest of pectate lyase E. Sample was prepared by adding 10 µl of a the PLe/AspN proteolytic reaction solution described in Materials and Methods to 140 µl of deionized water which was then injected onto a C_4 column (Vydac, Inc., 0.21 x 25 cm) and eluted with a 100 minute linear gradient of 0.1% TFA in 4% acetonitrile to 0.08% TFA in 56% acetonitrile. Peaks were detected using an inline UV detector set to 220 nm. The amino acid sequence of the 13 kDa peptide in peak 1 is given as deduced from the molecular weight and verified by amino acid sequencing.

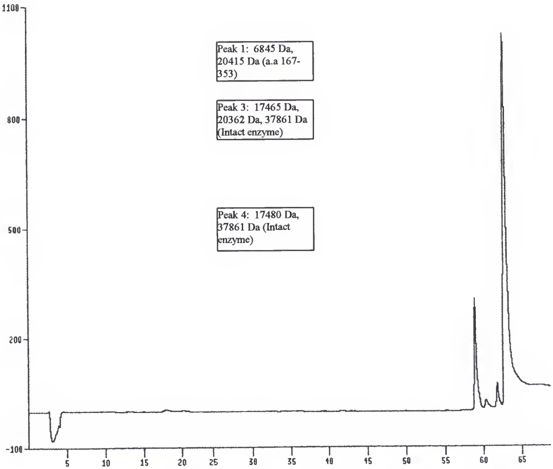


Figure 45: Reverse phase HPLC separation of a 3 hour GluC digest of pectate lyase C. Sample was prepared by adding 10 μl of a the PLc/GluC proteolytic reaction solution described in Materials and Methods to 140 μl of deionized water which was then injected onto a C_4 column (Vydac, Inc., 0.21 x 25 cm) and eluted with a 70 minute linear gradient of 0.1% TFA in 4% acetonitrile to 0.08% TFA in 56% acetonitrile. Peaks were detected using an inline UV detector set to 220 nm. The amino acid sequence of the 20 kDa peptide in peak 1 is given as deduced from the molecular weight and verified by amino acid sequencing.

stronger signal for the 17480 Da peptide as well as peptides with molecular weights of 5828 Da, 8727 Da and 9502 Da. Peak 1 was submitted for amino terminal amino acid sequencing.

Assignment of cut sites within each pectate lyase molecule

Using both the molecular masses seen in Figures 4, 5 and 6, as well as sequence information provided by the sequenced peptides from the HPLC separations, the cut sites within each molecule are shown in Table 1. The most susceptible peptide bond in pectate lyase B to proteolytic attack is that between Lys^{158} and Asp^{159}. Despite the structural homology expected to exist between PLb and PLc, pectate lyase C is not susceptible to proteolytic cleavage in this region. AspN cleaves the PLc molecule most readily between Leu^{193} and Asp^{194}, GluC cleaves the bond between Phe^{165} and Glu^{166}, and trypsin cleaves the peptide bond between Lys^{172} and Gly^{173}. Two loops are susceptible to proteolytic cleavage in PLe, with AspN and GluC cleaves the protein backbone at the Gly^{126}-Asp^{127} and Glu^{124}-Ser^{125} peptide bonds, respectively. Trypsin hydrolyzes the peptide bond between Lys^{164} and Asp^{165}, which is a loop structure analogous to that seen in the PLb and c structures.

Table 4: Proposed Amino Acid Sequences of Peptides Generated by Limited Proteolysis of Pectate Lyases B, C and E

Pectate Lyase B/AspN

Observed Mass (Da)	Proposed Sequence[a]	Calculated Mass (Da)[b]
16816	1-158	16626
21298	159-353[c]	21334

Pectate Lyase B/GluC

- No peptides observed

Pectate Lyase B/Trypsin

Observed Mass (Da)	Proposed Sequence[a]	Calculated Mass (Da)[b]
16740	1-158	16626
21550	159-353	21334

Pectate Lyase C/AspN

Observed Mass (Da)	Proposed Sequence[a]	Calculated Mass (Da)[b]
20201	170-353[c]	20075

Pectate Lyase C/GluC

Observed Mass (Da)	Proposed Sequence[a]	Calculated Mass (Da)[b]
17493	1-166	17382
20366	167-353[c]	20332

Pectate Lyase C/Trypsin

Observed Mass (Da)	Proposed Sequence[a]	Calculated Mass (Da)[b]
18054	1-172	17995
19862	173-353[c]	19718

Pectate Lyase E/AspN

Observed Mass (Da)	Proposed Sequence[a]	Calculated Mass (Da)[b]
13043	1-126[c]	12984
25235	127-355	25204

Pectate Lyase E/GluC

Observed Mass (Da)	Proposed Sequence[a]	Calculated Mass (Da)[b]
25444	125-355	25348

Pectate Lyase E/Trypsin

Observed Mass (Da)	Proposed Sequence[a]	Calculated Mass (Da)[b]
17377	1-164	17241
20993	165-355	20948

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[a] **Proposed sequences are based upon the molecular masses first seen to appear in the kinetic MALDI-TOF mass spectroscopy experiments. The persistence of these peptides through the 6 hour sampling period in the experiments was taken as an indicator of the high degree of susceptibility of these regions to proteolytic attack.**

[b] **Calculated masses are the average masses of the peptides based upon the given amino acid sequence.**

[c] **Sequence verified by amino acid sequencing.**

Discussion

Pectate lyase B was most susceptible to AspN, with 36% of the native enzyme population cleaved after 6 hours. In the electropherograms of samples taken from the reaction, only 4 peaks (two peaks, each with a shoulder) are observed, yet in the HPLC chromatograms, over 14 peaks are seen. This points to one of the deficiencies of the capillary electrophoretic technique, namely the high concentrations necessary to enable detection of component molecules in the sample, due to the small injection volumes used. However, the technique as applied here has allowed a kinetic profile of the proteolytic process to be determined with the simplicity and reliability gained by using an automated method. The relationship between the decrease in intact pectate lyase peaks areas and loss of enzymatic activity has been examined and the two have been found to be directly related (Hurlbert and Preston, 1998), with the decrease in intact pectate lyase peak area directly proportional to the loss in enzymatic activity.

Pectate lyase B was not susceptible to attack by GluC, and was the most resistant of the pectate lyases studied to proteolytic attack by trypsin, with 18% of the native PLb molecules serving as substrate for the endoprotease. Based upon the peptide masses seen in the kinetic MALDI-TOF MS experiments, and confirmed by amino acid sequencing, one region in PLb is uniquely susceptible to cleavage by the serine proteases used, that being the loop containing residues Asn^{151} through Ser^{166}. As seen in Figure 46A, aspartic acid residues are widely scattered across the surface of the protein, but Asp^{159} (Figure 46B, 46C) is recognized and the bond between it and Lys^{158} is cleaved by AspN more readily than any other aspartate. Trypsin, the other serine protease used in the study, is known to hydrolyze peptide bonds on the carboxy terminal side of either lysine and/or arginine, two amino acids that comprise 10% of the total amino acid content of the molecule (7% lysine, 3%

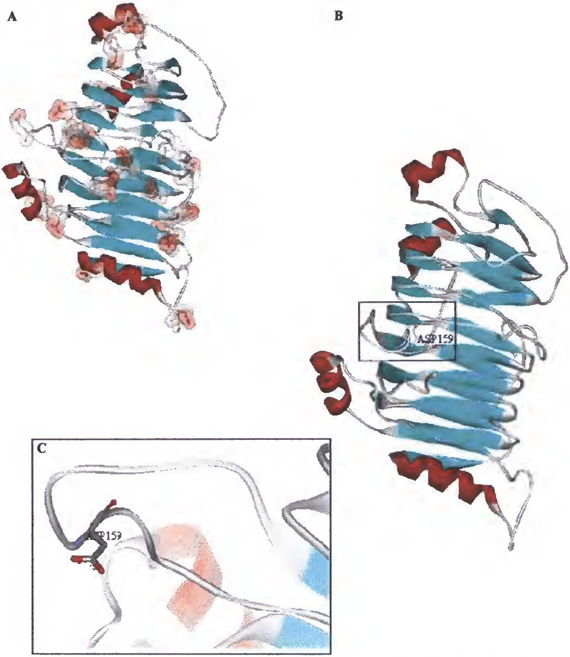


Figure 46: Molecular model of pectate lyase B. Depicted structure was homology modeled from the x-ray crystallographic structure of pectate lyase C on the SWISS-MODEL server (Peitsch, 1996). In Part A, the aspartate residues present in the protein are drawn as stick representations surrounded by a surface defined by the Van der Waals radius of the atoms in the side chain. In Parts B and C, the aspartate residue at the amino terminus of the peptide product generated in the initial cleavage of PLb by AspN, Asp^{159}, is shown. Figure was created using WebLab Viewer Pro ver 3.1 (Molecular Simulations Inc., San Diego, CA).

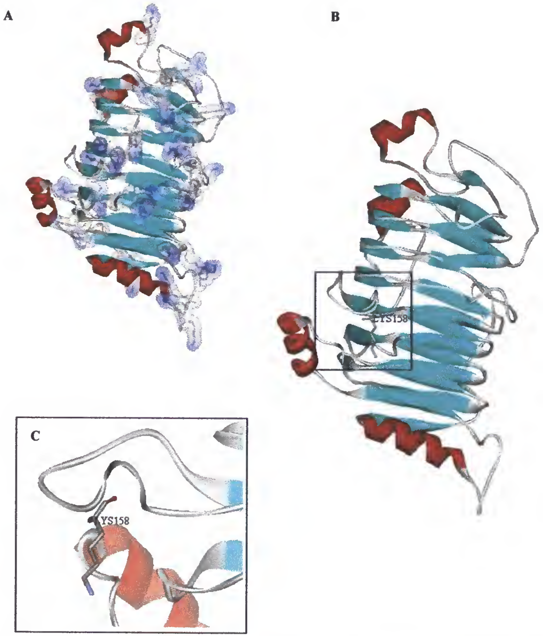


Figure 47: Molecular model of pectate lyase B. Depicted structure was homology modeled from the x-ray crystallographic structure of pectate lyase C on the SWISS-MODEL server (Peitsch, 1996). In Part A, the lysine and arginine residues present in the protein are drawn as stick representations surrounded by a surface defined by the Van der Waals radius of the atoms in the side chain. In Parts B and C, the lysine residue at the carboxy terminus of the peptide product generated in the initial cleavage of PLb by trypsin, Lys^{158}, is depicted. Figure was created using WebLab Viewer Pro ver 3.1 (Molecular Simulations Inc., San Diego, CA).

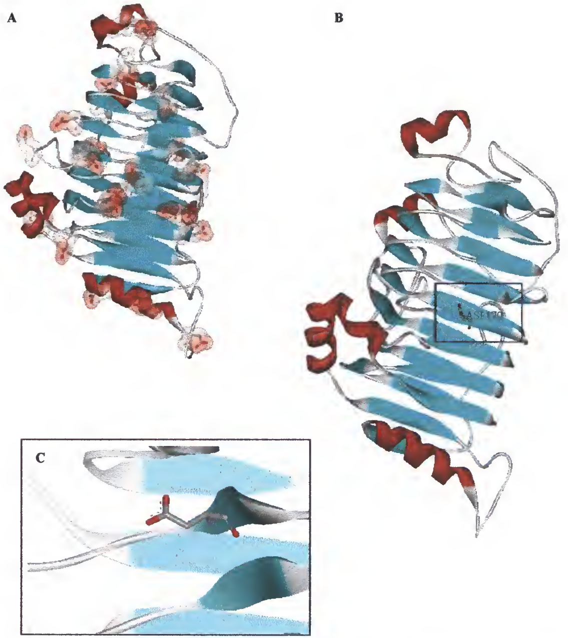


Figure 48: Molecular model of pectate lyase C based upon x-ray crystallographic data (Jurnak et al., 1993). In Part A, the aspartate residues present in the protein are drawn as stick representations surrounded by a surface defined by the Van der Waals radius of the atoms in the side chain. In Parts B and C, the aspartate residue at the amino terminus of the peptide product generated in the initial cleavage of PLc by AspN, Asp^{170}, is depicted. Figure was created using WebLab Viewer Pro ver 3.1 (Molecular Simulations Inc., San Diego, CA).

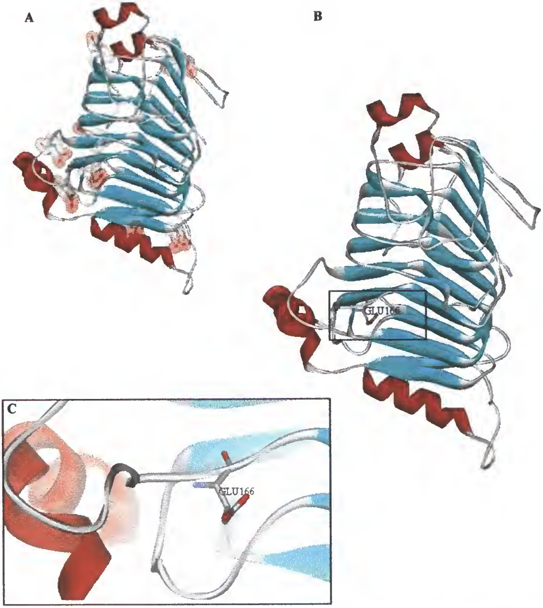


Figure 49: Molecular model of pectate lyase C based upon x-ray crystallographic data (Jurnak et al., 1993). In Part A, the glutamate residues present in the protein are drawn as stick representations surrounded by a surface defined by the Van der Waals radius of the atoms in the side chain. In Parts B and C, the glutamate residue at the carboxy terminus of the peptide product generated in the initial cleavage of PLc by GluC, Glu^{166}, is depicted. Figure was created using WebLab Viewer Pro ver 3.1 (Molecular Simulations Inc., San Diego, CA).

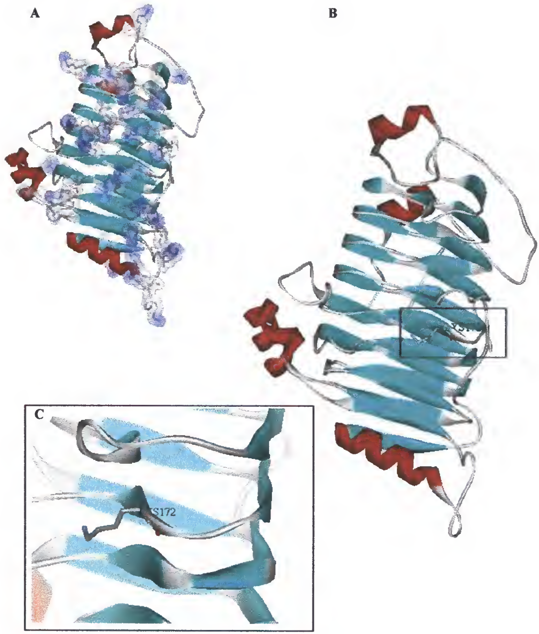


Figure 50: Molecular model of pectate lyase C based upon x-ray crystallographic data (Jurnak et al., 1993). In Part A, the lysine and arginine residues present in the protein are drawn as stick representations surrounded by a surface defined by the Van der Waals radius of the atoms in the side chain. In Parts B and C, the lysine residue at the carboxy terminus of the peptide product generated in the initial cleavage of PLc by trypsin, Lys^{172}, is depicted. Figure was created using WebLab Viewer Pro ver 3.1 (Molecular Simulations Inc., San Diego, CA).

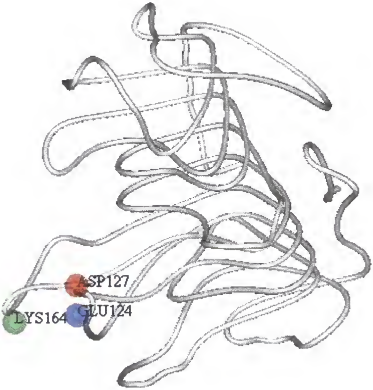


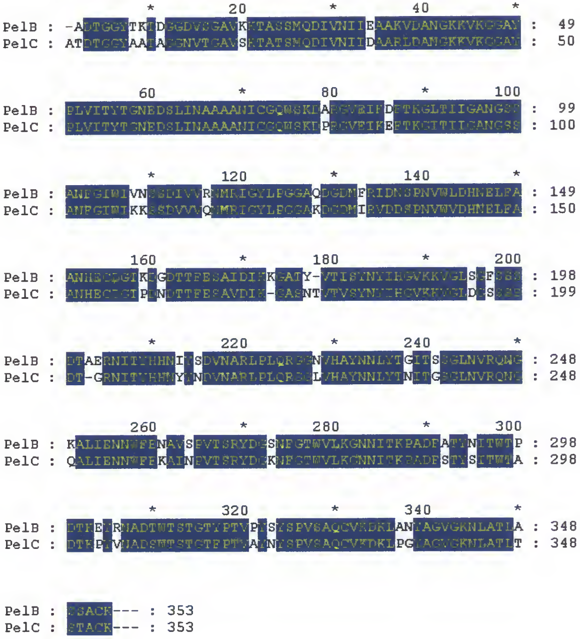
Figure 51: Molecular model of pectate lyase E based upon x-ray crystallographic data for the α-carbons of the polypeptide (Jurnak et al., 1993). The spheres at Glu^{124}, Asp^{127} and Lys^{164} represent the preferred cut sites of GluC, AspN and trypsin respectively. Figure was created using WebLab Viewer Pro ver 3.1 (Molecular Simulations Inc., San Diego, CA).

arginine) (Figure 48A). Trypsin hydrolyzes the same peptide bond in PLb as AspN does, as shown in Figure 48B and 48C. It is interesting to note that this bond is located on a loop covering the end of a putative substrate binding cleft that runs along the surface of the PLs. Any involvement of residues on this loop in substrate binding and/or release would indicate some degree of conformational flexibility possessed by the loop, an important factor in limited protease cleavage (Mihaly, 1992). While pectate lyase C may be the most resistant to proteolytic attack by AspN, it shares a high degree of susceptibility to degradation by both GluC and trypsin with PLe. Unlike PLb, the most susceptible bond for cleavage by AspN in PLc lies the fourth β-strand in parallel β-sheet 1. The bond between Val^{169} and Asp^{170} is preferentially cleaved by AspN (Figure 49), a peptide bond that lies on the same β-strand as the most susceptible peptide bonds cleaved by GluC and trypsin. The location of the AspN cleavage site implies that this β-strand may be spatially removed from the plane of parallel β-sheet 1, extending out into the solution. This is a distinct possibility given the observations from the x-ray crystallographic data that the portion of the protein containing the AspN cut site has a very short, two amino acid β-strand lying directly above it, and a twisted β-strand lying below it. The β-strand lying between two such protein segments would not be highly hydrogen bonded, and may jut out in to the solution environment. This hypothesis is further supported by the location of the most susceptible bond to trypsin cleavage, Lys^{172}-Gly^{173} (Figure 50). This bond lies on the carboxy terminal end of the β-strand that is cleaved by AspN. While the serine proteases used cleave peptide bonds within one of the β-strands comprising parallel β-sheet 1, the metalloprotease GluC preferentially cleaves the peptide bond between Glu^{166} and Ser^{167} (Figure 49). This bond is very close to the extended loop that contains the most susceptible peptide bonds for proteolytic cleavage in its homologue, PLb. These observations point to a key structural difference between pectate lyases B and C, namely the solution structure of the Asn^{151}-Ser^{166} loop present in both enzymes and the stretch of amino acids from Leu^{193} to Asp^{200}, also found in both proteins. The Asp^{170} recognized by AspN in PLc exists at position 169 in PLb. AspN does not preferentially cleave PLb between Ile^{168} and Asp^{169}, indicating that, structurally, these regions are significantly different in both PLb and PLc. This difference could be due to the behavior of last β-strand of parallel β-sheet 1, composed of amino acids Leu^{193} and Asp^{194} in PLc. This sheet is immediately followed by a turn; however, the turn region present in both enzymes is preceded by a serine in PLc and a phenylalanine in PLb, an amino acid with a side chain that would align such that the aromatic group was inside the β-helical core, thereby "anchoring" the turn. This single amino acid replacement in PLb may be enough to prevent the turn and the small strand sequence that precedes it from pushing away from the core of the molecule, something not seen in PLc and reflected in the susceptibility of the surrounding region to proteolysis by both serine proteases used.

The location of the initial cut sites in pectate lyase E are found in Figure 51, and are very straightforward with respect to interpreting the results. The Gly^{126}-Asp^{127} bond is most susceptible to AspN cleavage and it lies on a large loop extending away from the core helical structure. The Glu^{124}-Ser^{125} bond cleaved by GluC lies on the same loop. The Lys^{164}-Asp^{165} bond preferentially cleaved by trypsin lies on a loop that folds down to run parallel to the loop containing Asp^{127} and Glu^{124}, forming a prong-like structure far from the helical axis formed by the 3 parallel β-sheets. In the same regions of pectate lyases B and C, the loop containing Asp^{127} and Glu^{124} in PLe are α-helices in PLb and PLc. The loop containing Lys^{164} in PLe, however, is an analogous structure in the other two PLs, indicating a potential functional involvement of this loop structure in all of the pectate lyases.

CHAPTER 5
CONCLUSIONS

The ability of *Erwinia chrysanthemi* to effectively infect plant species is directly related to the battery of extracellular enzymes produced by the organism to degrade the structural polymers maintaining the cell walls of the host plant. The pectate lyases may be considered the advance guard in this phalanx of secreted enzymes, serving to degrade the outermost structural polymers and allowing access to the cellulose containing fraction of the plant cell wall. Underscoring the importance of the pectate lyases in the phytopathogenic process is the presence of so many isoforms of the enzymes and the structural genes encoding them on the bacterial chromosome. Two distinct gene duplication events have given rise to the major *pel* structural gene clusters found in the genome. In the time that has passed since those events, the structural genes comprising each cluster have independently evolved, with the most striking examples found in the *pelADE* cluster. In *Erwinia chrysanthemi* EC16, the *pelD* is truncated and non-functional and the other two gene products of the cluster have diametrically opposed isoelectric points (4.6 and 10.0 for PLa and PLe, respectively) and depolymerization profiles (random, and endolytic for PLa, whereas PLe is predominantly exolytic and processive). The gene products of the *pelBC* cluster on the other hand have not divergently evolved to as great an extent as evidenced by their shared identity (84%, Figure 52) and functional similarities under *in vitro* conditions. These observations suggest that the duplication event that gave rise to the *pelB* and *pelC* structural genes is much more recent or that the genes themselves are more stable. In either case, the organism has



```
                *        20         *        40         *
PelB : -ADTGGYTKTDGGDVSGAVKKTASSMQDIVNIIEAAKVDANGKKVKGGAY : 49
PelC : ATDTGGYAATAGGNVTGAVSKTATSMQDIVNIIDAARLDANGKKVKGGAY : 50

               60         *        80         *       100
PelB : PLVITYTGNEDSLINAAAANICGQWSKDARGVEIFDFTKGLTIIGANGSS : 99
PelC : PLVITYTGNEDSLINAAAANICGQWSKDPRGVEIKEFTKGITIIGANGSS : 100

                *       120         *       140         *
PelB : ANFGIWIVNSSDIVVRNMRIGYLPGGAQDGDMFRIDNSPNVWLDHNELFA : 149
PelC : ANFGIWIKKSSDVVVQNMRIGYLPGGAKDGDMIRVDDSPNVWVDHNELFA : 150

              160         *       180         *       200
PelB : ANHECDGTKDGDTTFESAIDIKKGATY-VTISYNYIHGVKKVGLSEFSSS : 198
PelC : ANHECIGTPDNDTTFESAVDIK-GASNTVTVSYNYIHGVKKVGLDESSSS : 199

                *       220         *       240         *
PelB : DTAERNITYHHNIYSDVNARLPLQRGGNVHAYNNLYTGITSSGLNVRQNG : 248
PelC : DT-GRNITYHHNYYNDVNARLPLQRGGLVHAYNNLYTNITGSGLNVRQNG : 248

              260         *       280         *       300
PelB : KALIENNWFENAVSPVTSRYDESNFGTWVLKGNNITKPADFATYNITWTP : 298
PelC : QALIENNWFEKAINPVTSRYDEKNFGTWVLKGNNITKPADFSTYSITWTA : 298

                *       320         *       340         *
PelB : DTKEYRNADTWTSTGTYPTVPYSYSPVSAQCVKDKLANTAGVGKNLATLA : 348
PelC : DTKPYVNADSWTSTGTFPTVAYNYSPVSAQCVKDKLPGTAGVGKNLATLT : 348

PelB : SSACK--- : 353
PelC : STACK--- : 353
```

Figure 52: Amino acid sequence alignment of pectate lyases B and C. Identical residues found in each sequence were determined using a Segment Pair Overlap search with a pairwise score cutoff value of 44. The alignment was produced with the MACAW program (Schuler et al., 1991).

evolved multiple enzymatic variations on the ancestral *pel* theme. This observation serves to indicate that the retention of multiple structural genes with independent control mechanisms is not to simply increase the copy number of the *pel* genes, allowing much more pectate lyase molecules to be produced for a given stimulus. A more plausible rationale may be that the multiple isoforms of pectate lyases have intrinsically different abilities, and the retention of these abilities gives the organism an edge in the pathogenic process that its forerunners did not have.

The experiments described in Chapter 2 have tested this theory by examining the functional differences that exist between the two pectate lyases sharing the highest degree of amino acid identity, PLb and PLc. Among the differences observed in the experiments, two characteristics were found to be shared by both PLb and PLc, namely their affinity for calcium ions and the extent to which they depolymerize the substrates they are presented. PLb and PLc share similar affinities for calcium ions, an ion required for eliminative catalysis of the α-1,4 glycosiduronosyl bonds in PGA. These results are in agreement with previous work (Hugouviex-Cotte-Pattat, 1997) showing similar calcium affinities for all of the pectate lyases, as is to be expected given the invariance of the residues thought to be involved in calcium binding as one looks at the amino acid sequences of all of the pectate lyases secreted by *Erwinia* species (Kita et al., 1996). In PLb and PLc secreted by *E. chrysanthemi* EC16, these residues are: Asp^{130} in PLb and Asp^{131} in PLc, Glu^{165} in Plb and Glu^{166} in PLc, and Asp^{169} in PLb and Asp^{170} in PLc (Figure 53). The observed affinities for Ca^{++} and the retention of the amino acids responsible for binding in the primary sequence of each enzyme indicate that the metal coordination sites in each enzyme are very similar, as predicted by the molecular model of PLb derived from the crystal structure of PLc. In addition to the similar calcium affinities displayed by both enzymes, the enzymes

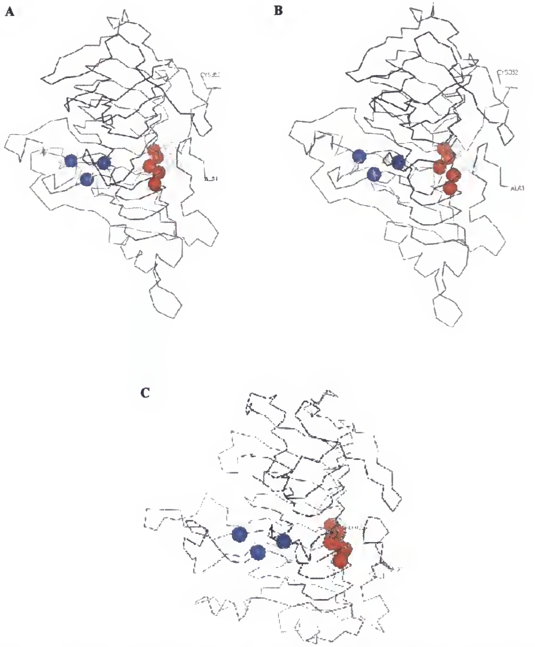


Figure 53: Molecular renditions of the α-carbon traces of pectate lyases B (A), C (B) and E (C). The amino acids implicated in Ca^{++} binding (Kita et al., 1996) have their α-carbons depicted as blue spheres, and those determined to be part of the vWiDH region (Henrissat et al., 1995) have α-carbons depicted as red spheres.

depolymerize methylated pectins to the same extent. It is not known if the methyl ester substitution on the carbonyl carbon of the galacturonic acid ring interferes with resonance stabilization of the reaction intermediate, or in the binding of the pectin molecule by the enzyme. Given the conformational shifts observed for the pectins, it is conceivable that pectin molecules adopt a conformation far different from polygalacturonic acid molecules, thereby preventing the pectate lyases from effectively binding and depolymerizing the polymers. However, pectate lyase C has a much higher initial activity towards methylated substrates than PLb, and ^{1}H-NMR and HPLC data has shown that PLc can more readily bind and cleave these substrates than PLb. The products formed during the reaction contained methylated constituent residues at all positions other than the non-reducing terminus. This indicates that while the placement of a C-6-O-methyl galacturonic acid moiety in the reducing terminal position of the bond to be cleaved does not allow the lyase catalyzed reaction to proceed, methyl esterification of residues towards the reducing terminus of the substrate molecule does not inhibit binding of the potential substrate.

Beyond these observed similarities, the remainder of the kinetic data obtained in the experiments serve to identify key functional differences that have arisen between the two proteins. Pectate lyase B has now been shown to be a more endolytic enzyme than PLc when presented with PGA in a calcium-cross bridged state. This tendency was hinted at in previous work (Hurlbert, 1996), but not definitively demonstrated. PLb has a much higher K_m and obtainable V_m for cross bridged PGA than does PLc. In theory, this is attributed to the ability of PLb to bind both PGA duplex structures formed by substrate binding of calcium ions, as well as pre-gel aggregates of these duplex molecules. Both enzymes show similar initial activities when challenged with these substrates, but PLb produces more detectable trimer and tetramer molecules in the initial hours of reaction than PLc under all conditions studied, suggesting that PLb has no difficulty binding and cleaving substrates that have not only formed calcium-cross bridged duplex structures, but also begun to aggregate. PLc, on the other hand, may initially find stretches of non-duplexed/non-aggregated PGA molecules that can be readily cleaved, but after these initial cleavages are made, the enzyme has a much more difficult time binding and cleaving further substrate molecules. In the absence of preincubation, PLc shows a slightly higher initial activity than PLb, suggesting that under conditions of low or zero aggregation, the PGA duplexes that form quickly in solution can be readily cleaved by the enzyme. The advantage shown by PLb over PLc when dealing with polygalacturonic acid and the secondary structures formed by the polymer in the presence of calcium is not shown when methylated substrates are presented to the enzyme. In this situation, the substrate has formed a duplex (or higher) structure with the driving force behind the association event being hydrophobic interactions of the methyl groups substituted onto the carbonyl oxygen. In this situation, the non-methyl esterified residues may be kinked out along the chain (Rees, 1982), representing a conformation that PLb does not bind as effectively as PLc does. In fact, PLc shows the highest initial velocities towards substrates that have formed a hydrophobically driven duplex structure, but have not had sufficient time to form calcium mediated cross-bridges between adjacent stretches of non-methylated galacturonic acid residues.

These functional differences may be particularly significant given the complex nature of the plant cell wall and intracellular matrix material (Fry, 1986) and the importance of the pectate lyases in the pathogenic process. The pectate lyases are produced as the cell enters the leaf tissue, whether through the stomates or physical breaks in the tissue itself. Once in the mesophyll, the resultant removal of nitrogen limiting conditions, as well as the decreased medium osmolarity and iron and oxygen concentrations cause an immediate induction of all pectate lyase genes. The production and secretion of these enzymes results in formation of 2-keto-3-deoxygluconate, di- and tri- galacturonides that serve as inducers of cellulases, pectate hydrolases, pectin lyases, pectinases, and pectate hydrolases, and the maceration process continues in earnest. The products of the *pel* genes must serve as the advance guard in this process though, and be able to initially macerate the various types of polymers presented to them when the cell first invades the host. By producing two enzymes that can degrade not only PGA, but layers of PGA molecules that have formed duplex structures due to the presence of calcium ions (essentially aggregates) or hydrophobic associations, the cell is insured of striking a rapid blow to the host. This opportunity can then be immediately followed by the more specialized enzymes secreted in the second wave, ultimately resulting in the degradation of the primary cell wall and the rupture of the (Mussell and Strand, 1976).

The functional benefits gained by the organism in the evolution and retention of multiple pectate lyases are only a part of the story however. The data obtained in the denaturation experiments described in Chapter 3 point to the low overall molecular stability of the pectate lyases. Pectate lyases B and E are completely denatured in 2.0 M guanidine hydrochloride, and PLc is apparently in a second structural transition state when exposed to GdnHCl at this concentration. None of the lyases displays appreciable activity at GdnHCl concentrations higher than 1.5 M. This low tolerance for chemical perturbation is surprising given not only the fact that these are extracellular enzymes, but also in comparison to a sampling of other published protein stabilities (Eichler et al., 1994, Szeltner and Polgar, 1996, Zhuang, et al., 1996, Rall et al., 1998, Schlidbach et al., 1998, Wang et al., 1998), where the midpoints of denaturation are reached at GdnHCl concentration in the range of 1.5 to 3.2 M, not the 0.75 M to 1.75 M range seen in the pectate lyases. The ease of unfolding can be explained however, given the published three-dimensional structures of the proteins. Upon addition of the chaotrope, the hydrogen bonds between adjacent strands of the core β-sheets is disrupted, and the structural perturbations that follow may allow access of both denaturant and solution into the hydrophobic helical core (Peterson et al., 1997). When this happens, it is not difficult to envision a secondary disruption of structure caused by the exposure of the hydrophobic side chains to the solvent and salt. This could explain the pronounced two state transition seen in PLc, and to a lesser extent PLb and PLe. All of the pectate lyase samples studied displayed the same molecular ellipticity upon completion of the denaturation reaction (by 4 M GdnHCl), suggesting that all reach the same structural state (or lack thereof).

Preliminary refolding data indicates that the ease with which the pectate lyases can be denatured does not translate into rapid refolding of the protein, thereby preventing a rigorous thermodynamic analysis of the data from the refolding reaction (Gittis et al., 1994; Santoro and Bolen, 1988a; Santoro and Bolen, 1988b). The proper alignment of the component β-strands in the protein must therefore not be easily accomplished in solution without the aid of some component found in the periplasm of the pathogen. This is supported by previous data (Henrissat et al., 1995) implicating a conserved amino acid sequence in all of the pectate lyases, termed the vWiDH region, in the proper folding and/or secretion of the pectate lyases (aa 140-144 in PLb, aa 141-145 in PLc, aa 144-148 in PLe) (Figure 53).

A better test of both the environmental survivability and structural properties of the β-helical topology can be found in the exposure of the pectate lyases to varying temperatures. The data obtained from these experiments point out the differential stabilities of the three major pectate lyases secreted by *E. chrysanthemi* EC16, with PLb being the most stable and PLe being the least stable. Pectate lyase B also showed the largest extent of thermal activation, with this observed activation coming at a temperature just before the melting transition of the enzyme. Pectate lyase C, on the other hand, shows a much smaller extent of activation, and that coming 5°C lower than the observed commencement of the melting transition for the enzyme. Unlike PLb and PLc, pectate lyase E shows no thermal activation, but may thermally denature through an somewhat stable intermediate state, as supported by the change in slope of the Θ_{MRW}^{222nm} versus temperature from 40 °C to 55°C. The CD and activity data provide a view into the potential differences that may be evolving between the lyases. The immediate and continuous loss of activity seen in PLe at all temperatures higher than 30°C would indicate that a finite spatial arrangement of either active site or substrate binding residues exists, and that applying energy to the entire system displaces these residues with an immediate decrease in activity. Using an analogy gleaned from the glycosyl hydrolases (Davies and Henrissat, 1995), this arrangement would be analogous to a tunnel type of active site topology. Cellobiohydrolase II possesses such a topology and has been shown to processively degrade cellulose polymers with cellobiose formed as the predominant product, much the same as PLe does with PGA. The crystal structure of PLe does not show a loop closed over the active site cleft of the enzyme, but it is conceivable that the extended loop seen near the active site cleft in the enzyme (Yoder et al., 1993) is in a much more closed state than it is in PLb or PLc. This type of structural arrangement would not prohibit the enzyme from making endolytic cleavages, which PLe has been demonstrated to make (Preston et al., 1992), since the active site cleft is not completely closed. A semi-closed structure would also be more susceptible to thermal inactivation than a much more open binding cleft type of active site, as is seen in the thermal denaturation experiments. This type of structural arrangement could also help to explain the lack of substrate protection afforded the enzyme in preliminary experiments exposing PLe to the site specific protease trypsin (Preston, Unpublished results). Along these lines, it is also conceivable that the difference in endolytic cleavage ability seen between PLb and PLc could be due to a slight pinching off of the active site cleft in PLc due to a loop closure. This could also explain the difference in temperature optimum and structural state seen in the PLc thermal denaturation experiments. Pectate lyase B has been shown to be more endolytic than either of the other enzymes, behavior typical of an open cleft type of active site topology. The marked increase in activity seen for PLb at increasing temperature could also be explained by the enzyme possessing a more open, or cleft, type of active site topology than the other pectate lyases, since neither the binding nor catalytic steps of the reaction rely upon residues located on a loop comprising an edge of the cleft. This is not to say that the active site topology of PLb is strictly a cleft or groove, that of PLc is a partial tunnel and that of PLe is a tunnel. The degree of structural separation in active site topologies of the three enzymes is slight, but very real given the observations made, and best explained in the terms presented.

The differences in structural stability seen between PLs b, c and e are mirrored in the sensitivities to proteolytic attack and the most susceptible peptide bonds to proteolytic cleavage. It has been previously shown that the PLs are inactivated by proteolytic cleavage (Hurlbert, 1996) and that the initial cleavage events at the most susceptible sites in the protein are responsible for the observed decrease in activity (Hurlbert and Preston, 1998). These observations are clear in light of the location of the susceptible peptide bonds in each protein; these bonds are found in or adjacent to the charge groove thought to be involved in substrate binding. Cleavage of these bonds essentially cuts the protein in half, splitting the groove and/or breaking apart the reaction center of each protein.

The most susceptible peptide bonds in PLb and PLc to proteolytic scission are not found in similar regions of each molecule, and suggest that significant differences in the solution structure of the homologues exists. A single peptide bond in PLb is uniquely susceptible to proteolytic attack, and that bond is part of a loop thought to cap the substrate binding groove and/or the catalytic residues of the protein. The same loop exists in PLc, as does the aspartate residue found in PLb on the loop. However, the lysine found to precede the aspartate in the PLb sequence is replaced by a proline residue in the PLc sequence. This may block the protease from binding to the protein backbone in the region, as well as forcing the loop to exist in a more closed conformation relative to the bulk of the enzyme. Pectate lyase C is cleaved in the same the region by GluC, between Glu^{166} and Ser^{167}. This peptide bond is at the base of the loop, just prior to the amino terminus of the fourth β-strand comprising parallel β-sheet 1. The location of the site may be sufficiently spaced from the loop region to allow cleavage the Glu^{166}-Ser^{167} peptide bond, which is interesting when looking at the other cut sites in the protein, all of which are much more internal than in the other two enzymes. This may reflect a more dynamic internal structure, which is supported by the relative ease of which PLc is denatured with guanidine hydrochloride.

The lack of any type of internal proteolytic cleavages supports the theory that PLe has a different solution structure/active site topology than PLb or PLc. The cut sites in the pectate lyase E molecule are located on two adjacent loops extruding far from the main body of the enzyme. This is relevant when considering that the peptide bonds cleaved during limited proteolysis are usually found in solution accessible or otherwise dynamic sequences of amino acids (Mihalyi, 1978). The β-helical core of the enzyme serves as one cleft wall and the two loop sections form another wall of a deep binding cleft, which only exposes the two loops and the periphery of the protein to a protease present in solution. As seen in the GluC and trypsin digestions of PLe, removal of these extended loop regions and the amino terminal portion of the protein results in multiple proteolytic cleavages along both halves of the protein.

The demonstrated susceptibilities of the pectate lyases to proteolytic attack indicate that the proteins did not evolve in the face of resistance from the host cells involving proteases. Plant cells possess proteases, but their primary role is not to defend the host from pathogenic invasion (Boller, 1986), but rather in metabolic maintenance of the plant cells. The exception to this case may be the rupture of vacuoles in the plant cell following the protoplast disruption accompanying mechanical failure of the plant cell wall. In this case endoproteases do exist that would be able to degrade the extracellular enzymes secreted by the invading pathogen (Storey, 1986), but by that point, the job of those virulence factors is done, and a successful attack has been launched against the host organism.

In conclusion, there may be several reasons for the evolution and retention of multiple isoforms of pectate lyases. The present work has shown that the β-helical folding motif does not impart a high degree of structural stability to the proteins. The enzymatic function of the enzymes is directly tied to the β-helical fold, and even slight perturbations of this core structure serve to remove observable activity from the samples tested. The core β-helical structure of the pectate lyases may have evolved to facilitate correct folding in the periplasm, followed by rapid secretion, allowing the infectious process to occur smoothly (Henrissat et al., 1995). These observations indicate that the presence of multiple versions of the ancestral *pel* protein may be a way to increase the number of enzyme molecules secreted in response to a given stimulus, thereby allowing the organism to strike at least some sort of blow to the host tissue before the inactivation of the proteins by environmental factors.

Despite the lack of structural stability possessed by the pectate lyases, the organism has apparently retained the structural genes for multiple extracellular enzymes with a common folding motif that, with only a small amount of evolutionary divergence, have evolved discrete structural differences between themselves. The sensing and control systems that have evolved allow the organism to detect when it has entered an arena necessitating the increased production of the pectate lyases. Once produced and secreted, the finite structural differences present in the different isozymes impart to the organism the potential to successfully degrade a wider array of host cell tissues with a limited number of secreted enzymes. These enzymes and their success in degrading different types of host tissue in turn allows rapid production of the primary inducer (2-keto,3-deoxygluconate) of the remaining enzymes involved in the pathogenic process. Upon host cell rupture, the pectate lyases, as well as the other enzymes secreted by the pathogen, may be degraded by proteases present in the vacuoles of the plant cell, but by this time, the constraints of enzyme production within the *Erwinia chrysanthemi* EC16 cell are gone and the released proteases may be evaded by the motile pathogen, or simply overwhelmed by the amount of protein that the pathogen can produce and secrete. In either case, the pathogen has succeeded in establishing itself, at least temporarily, within the host tissues, thanks in part to the presence of multiple isoforms of the pectate lyases.

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BIOGRAPHICAL SKETCH

Jason Christopher Hurlbert was born in Jacksonville, Florida on October 22, 1970. He attended San Pablo Public Elementary School in Neptune Beach, FL, and St. Andrews Episcopal Day School in Jacksonville until the seventh grade. He applied for admission to the Episcopal High School of Jacksonville and was not only admitted but received an academic scholarship to attend. During his high school years, he worked as a supermarket stockboy, automotive detailer, automotive repossession agent, assistant parts manager, and computer programmer. In 1988, he graduated from Episcopal High School of Jacksonville as a National Merit Scholar. He eagerly attended the University of Florida in August of 1988 and supplemented his scholarship income by working as a pizza delivery driver, pizza chef, janitor, gun range attendant, and construction worker. Despite the variety of work experience accrued during his short life, during his fourth year of college, he was still undecided about his future. He began a senior research project in the area of biochemistry and microbiology in Dr. James F. Preston's laboratory in the spring of 1992. This introduction to laboratory research sparked a desire to pursue graduate study in microbiology. He received his Bachelor of Science in Agriculture degree from the Department of Microbiology and Cell Science in the spring of 1993 and was accepted to graduate school in the same department in the fall of 1993.

His graduate research has been focused on the biochemical analysis of the homologous pectate lyase enzymes produced by the phytopathogen *Erwinia chrysanthemi* EC16. The enzymes have been found to possess a unique three dimensional structure, and one of the primary aspects of his work has been investigating the link between this structure and the catalytic function of the proteins. He plans to continue postdoctoral studies in the structure/function relationship of proteins and eventually hopes to secure an academic position.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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Distinguished Professor of Microbiology and
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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

May, 1999

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